The *Bacteroides* NBU1 Integrase Performs a Homology-independent Strand Exchange to Form a Holliday Junction Intermediate*

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The *Bacteroides* mobilizable transposon NBU1 uses an integrase (IntN1) that is a tyrosine recombinase for its integration and excision from the host chromosome. Previously we showed that IntN1 makes 7-bp staggered cuts within the NBU1 *att* sites, and certain mismatches within the crossover region of the *attN1* site (G(−2)C *attN1*) or the chromosomal target site (C(−3)G *attBT1-1*) enhanced the *in vivo* integration efficiency. Here we describe an *in vitro* integration system for NBU1. We used nicked substrates and a Holliday junction trapping peptide to show that NBU1 integration proceeds via formation of a Holliday junction intermediate that is formed by exchange of bottom strands. Some mismatches next to the first strand exchange site (in reactions with C(−3)G *attBT1-1* or G(−2)C *attN1* with their wild-type partner site) not only allowed formation of the Holliday junction intermediate but also increased the rate of recombinant formation. The second strand exchange appears to be homology-dependent. IntN1 is the only tyrosine recombinase known to catalyze a reaction that is more efficient in the presence of mismatches and where the first strand exchange is homology-independent. The possible mechanisms by which the mismatches stimulate recombinant formation by IntN1 are discussed.

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The *Bacteroides* mobilizable transposon, NBU1, can be transferred from one cell to another via conjugation, using transfer proteins provided in *trans* by a coresident conjugative transposon, CTnDOT (1). Using its own integrase IntN1, NBU1 integrates into the host chromosome in the recipient cell, at a specific site called *attBT1-1*, located at the 3′-end of a *leu* tRNA gene (2). IntN1 carries the conserved residues RKHRHY that are characteristic of tyrosine recombinases (2), and substitution of each of these residues inactivates the protein (3). The target *attBT1-1* contains a 14-bp “common core” sequence, which is identical to a sequence on the NBU1 *attN1* and gets duplicated following integration (4). IntN1 makes 7-bp staggered cuts within this 14-bp region on the top and bottom strands (3) (Fig. 1A). Thus, similar to other characterized tyrosine recombinases, recombination by IntN1 occurs within a specific core sequence, which is identical in the two substrates.

Tyrosine recombinases such as phage λ integrase and Cre and Flp recombinases have an absolute requirement for homology within the crossover regions (5−10). According to the current “strand-swapping isomerization” model, strand exchange occurs by cleavage of partner strands in each site forming a 3′-phospho-tyrosyl bond and free 5′-OH groups. Two or three bases are melted from each strand and annealed to the complementary strand in the partner (11). The bases are tested for homology with the partner by effective base-pairing before ligation (11, 12) to form a Holliday junction intermediate. Isomerization of the intermediate leads to the second round of cleavage and strand exchanges at the opposite end of the crossover region, where effective base pairing is again required to form complete recombinants.

Further analysis suggested that the homology between the NBU1 *att* sites was not critical to recombination and probably existed only to preserve the *leu*-tRNA gene sequence. Mismatches were introduced in the NBU1 core region in either *attN1* or *attBT1-1*, and the mutant *att* sites were tested for their *in vivo* integration efficiency (13). When the bases A(−6) and G(−7) next to top strand cleavage site (Fig. 1A) were mutated in either *att* site, integration efficiency was not affected significantly. Substitutions of bases A(−6), C(−5), and C(−4) decreased the integration frequencies to different extents, but the recombination was not restored to wild-type levels when the same mutation was present on both the *att* sites. The effect of the G(−2)C and C(−3)G substitutions were more surprising. The C(−3)G mutation enhanced *in vivo* integration frequencies by 100-fold, but only when it was placed in the *attBT1-1* site (Fig. 1B). When placed in the *attN1* site the integration was adversely affected. On the other hand, the G(−2)C mutation in *attN1* stimulated integration by 300-fold, but abolished activity when placed in *attBT1-1* (Fig. 1B) (13). IntN1 is the only tyrosine recombinase known whose activity is stimulated by mismatches within the crossover region.

It has not been demonstrated that the recombination mechanism for any mobilizable transposon using a tyrosine recombinase involves a Holliday junction intermediate. We have developed here an *in vitro* integration assay for NBU1, and we report that NBU1 recombination proceeds via formation of a Holliday junction (HJ) intermediate. IntN1 differs from other tyrosine recombinases, such as Flp and Cre, in that it has an absolute requirement for homology at the sites of recombination, and its integration is stimulated by mismatches in the partner sequence.

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<sup>2</sup> The abbreviations used are: HJ, Holliday junction; Cm, chloramphenicol; Tp, trimethoprim; Ap, ampicillin; Kn, kanamycin; Int, integrase; IHF, integration host factor.
binases, because the HJ intermediate is formed even in the presence of mismatches next to the first strand exchange site.

**EXPERIMENTAL PROCEDURES**

**Media, Proteins, and Plasmids—**All *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or LB-agar plates. The antibiotic concentrations used were as follows: ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (20 μg/ml), and trimethoprim (100 μg/ml). Plasmid preparations were performed with Qiagen kits. Native IntN1 was expressed from a T7 promoter in *E. coli* BL21(DE3)pLysS strain and purified as described previously (14). Oligonucleotides were obtained from IDT. [γ-32P]ATP and T4 polynucleotide kinase and removing unincorporated label using a G50 mini-Sephadex column. The labeled oligonucleotide was then mixed with the complementary oligonucleotide at a molar ratio of 1:5 in 10 mM Tris-HCl, pH 8.0, 0.1 mM KCl, 5 mM EDTA, and annealed by heating to 95 °C for 2 min followed by slow cooling. Oligonucleotides used for wild-type and mutant *attBT1*-1 substrates are shown in Table 1. Substrates carrying a nick at one of the cleavage sites were prepared by annealing together three oligonucleotides (Table 1), one of which was 5'-radiolabeled.

In Vitro Recombination Assay—The plasmid pJSW200 was previously constructed by cloning the NBU1 *attN1* site into the pGEM-T vector (13). Assays were performed in 20-μl volumes containing 1.5 μg of supercoiled pJSW200 (0.6 pmol) and 1.5–2 pmol of radiolabeled *attBT1*-1 (44 bp) in 50 mM Tris-HCl, pH 8.0, 70 mM KCl, 10% glycerol, 1 mM EDTA, 0.2 mg/ml bovine serum albumin, 5 mM dithiothreitol, and 5 mM spermidine. Approximately 1 μg of the integrase IntN1 (18 pmol) and 0.2 μg of purified *E. coli* IHF were added. Peptide-supplemented reactions had 1 μl of the peptide diluted appropriately in water. The reactions were incubated at 37 °C for 2 h unless otherwise stated and were stopped by the addition of SDS to a final concentration of 1%. The reaction products were analyzed by electrophoresis through a 1% agarose gel in 1 X TBE (8 mM Tris borate, 2 mM EDTA) buffer.

Restriction Analysis of Recombinant Products—The *in vitro* recombination reactions were set up as described above, in 5-μl volumes. After incubation at 37 °C for 2 h, the reactions were extracted with phenol-chloroform-isooamyl alcohol, the DNA was precipitated and digested with Scal enzyme (Invitro-

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**FIGURE 1.** A, the common core region of NBU1 *att* sites. The bases are numbered 1 through 14. IntN1 makes 7-bp staggered cuts as shown by the black arrows. Only the top strand is shown. B, effect of G(-2)C and C(-3)G substitutions on integration in vivo. ++, wild-type frequency; ++, intermediate frequency; +++++, 100-fold higher than wild-type. Adapted from Ref. 13.

**TABLE 1**

Oligonucleotides used to prepare *attBT1*-1 substrates

| Oligonucleotide | Sequence |
|-----------------|----------|
| WT ts           | 5'-TCTTACGGGGGACCCGGGTCGCTCAGACCCTGGGATG-3' |
| WT bs           | 5'-CCTCCTCCTGACGGGGGTAGCTCAGACCCTGGGATG-3' |
| C(-3)G ts       | 5'-TCTTACGGGGGACCCGGGTCGCTCAGACCCTGGGATG-3' |
| C(-3)G bs       | 5'-CCTCCTCCTGACGGGGGTAGCTCAGACCCTGGGATG-3' |
| A(-8)T ts       | 5'-TCTTACGGGGGACCCGGGTCGCTCAGACCCTGGGATG-3' |
| A(-8)T bs       | 5'-CCTCCTCCTGACGGGGGTAGCTCAGACCCTGGGATG-3' |
| G(-7)C ts       | 5'-TCTTACGGGGGACCCGGGTCGCTCAGACCCTGGGATG-3' |
| G(-7)C bs       | 5'-CCTCCTCCTGACGGGGGTAGCTCAGACCCTGGGATG-3' |
| G(-2)C ts       | 5'-TCTTACGGGGGACCCGGGTCGCTCAGACCCTGGGATG-3' |
| G(-2)C bs       | 5'-CCTCCTCCTGACGGGGGTAGCTCAGACCCTGGGATG-3' |
| Nick ts left    | 5'-TCTTACGGGGGACCCGGGTCGCTCAGACCCTGGGATG-3' |
| Nick ts right   | 5'-CCTCCTCCTGACGGGGGTAGCTCAGACCCTGGGATG-3' |
| Nick bs right   | 5'-CCTCCTCCTGACGGGGGTAGCTCAGACCCTGGGATG-3' |
| Nick bs left    | 5'-CCTCCTCCTGACGGGGGTAGCTCAGACCCTGGGATG-3' |

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from PerkinElmer Life Sciences. T4 polynucleotide kinase was obtained from Fermentas.

Preparation of Radiolabeled *attBT1*-1 Substrates—The *attBT1*-1 substrates were prepared by first 5'-radiolabeling one of the oligonucleotides using [γ-32P]ATP and T4 polynucleotide kinase and removing unincorporated label using a G50 mini-Sephadex column. The labeled oligonucleotide was then mixed with the complementary oligonucleotide at a molar ratio of 1:5 in 10 mM Tris-HCl, pH 8.0, 0.1 mM KCl, 5 mM EDTA, and annealed by heating to 95 °C for 2 min followed by slow cooling. Oligonucleotides used for wild-type and mutant *attBT1*-1 substrates are shown in Table 1. Substrates carrying a nick at one of the cleavage sites were prepared by annealing together three oligonucleotides (Table 1), one of which was 5'-radiolabeled.
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**FIGURE 2. Mating assay to test for integration efficiencies of top versus bottom strands of attN1.** The donor strain carries the pir-dependent pEPE:attN1 plasmid with the NBU1 attN1 site cloned in either the forward or the reverse orientation. The pir recipient cell has the target attBT1-1 site in the chromosome and the plasmid pIntN1 expressing the NBU1 Int. Donors and recipients are mixed together for conjugation to occur. Transconjugants that are resistant to ampicillin (Ap), kanamycin (Kn) and chloramphenicol (Cm) arise only by integration of the pEPE:attN1 plasmid into the recipient chromosome.

Denaturing Agarose Electrophoresis—To analyze the recombination products by denaturing electrophoresis, the reactions were set up as described above, but they were stopped by adding NaOH to a final concentration of 50 mM. The products were run on a 1% alkaline agarose gel (50 mM NaOH, 5 mM EDTA) in alkaline buffer (50 mM NaOH, 5 mM EDTA) at 30 V for 16 h. The gel was dried and exposed as described above.

Two-dimensional Gel Analysis of Recombination Reactions—Recombination reactions supplemented with the peptide were set up in 2× volumes. Two 15-μl samples were electrophoresed through a 1% agarose gel in 1× TBE. One lane from each pair was sliced out of the gel and prepared for the second dimension as described below; the rest of the gel was treated and exposed to phosphorimaging screens. The gel slices were soaked in alkaline buffer (50 mM NaOH, 5 mM EDTA) for 1 h, and then embedded in a tray with 200 ml of a molten agarose solution in 50 mM NaOH, 5 mM EDTA. The gel was then electrophoresed in alkaline running buffer (50 mM NaOH, 5 mM EDTA) at 30 V for 16 h. The gel was then dried and exposed to phosphorimaging screens.

In Vivo Recombination Assay—The E. coli strains used were previously constructed (13). The donor strain with the attN1 site in the reverse orientation was constructed in this study. Primers carrying the restriction sites for Xhol and XbaI were designed and used to amplify the attN1 gene from pJWS200. The attN1 site was then cloned into the pir-dependent pEPE vector that was cut with Xhol and XbaI enzymes. After transformation into E. coli BW19851 cells, chloramphenicol-resistant colonies were selected, and the inserts were sequenced to check for the presence of the attN1 site.

An E. coli mating assay was used as previously described (3, 13). Donor strains are resistant to chloramphenicol (Cm) and trimethoprim (Tp), and recipient strain is resistant to ampicillin (Ap) and kanamycin (Kn). Recipient colonies were selected for growth on LB Ap Kn plates, and integrant colonies were selected on LB Ap Kn Cm plates. The integration frequency was calculated as the ratio of the number of Ap Cm Kn-resistant colonies to the number of Ap Kn-resistant colonies. A colony screening was utilized on the integrant colonies to determine if they were obtained by site-specific integration of attN1 into attBT1-1. We used primers that anneal to attBT1-1 and the regions flanking attN1, that such amplification of attBT1-1, attN1, attR-N1, or attL-N1 gave different sized products (3, 13).

**RESULTS**

Does IntN1 Use a Single-stranded or Double-stranded Substrate?—The Vibrio cholerae CTX phage uses the host-encoded XerC/XerD recombinases to integrate into the host chromosome. The single-stranded DNA genome of CTXφ folds into a hairpin structure that creates a recombination site for XerC/XerD (16). Only one pair of strand exchanges is performed, and the resulting HJ intermediate is converted to double-stranded DNA by cellular processes. The integration of integron gene cassettes mediated by the tyrosine recombinase IntI1 also uses a folded single-stranded attC substrate (17). The active hairpin structure is formed only by one of the single strands: only the (+) strand of CTXφ or the attC bottom strand of the integron cassette can integrate. Because single-stranded DNA is part of the natural lifecycle in conjugal and mobilizable transposons, it is conceivable that the single strand that enters the recipient is the actual substrate instead of the complementary double strand form. Therefore, we used an E. coli in vivo conjugation assay (13) to test if IntN1 shows a preference for top versus bottom strand of the NBU1 attN1 (Fig. 2).

The recipient strain has the Bacteroides target attBT1-1 site integrated in the chromosome and expresses the NBU1 intN1 gene on a plasmid. The donor strain carries a conjugative pir-dependent Cm-resistant plasmid (pPE:attN1) with the NBU1 attN1 site. Following conjugation, ApR, KnR, and CmR colonies were selected, and the integrant colonies were analyzed by gel electrophoresis as described above. The products were set up as described above, but they were stopped by adding NaOH to a final concentration of 50 mM. The products were run on a 1% alkaline agarose gel (50 mM NaOH, 5 mM EDTA) in alkaline running buffer (50 mM NaOH, 5 mM EDTA) at 30 V for 16 h. The gel was then dried and exposed to phosphorimaging screens. The gel slices were soaked in alkaline buffer (50 mM NaOH, 5 mM EDTA) for 1 h, and then embedded in a tray with 200 ml of a molten agarose solution in 50 mM NaOH, 5 mM EDTA. The gel was then electrophoresed in alkaline running buffer (50 mM NaOH, 5 mM EDTA) at 30 V for 16 h. The gel was then dried and exposed to phosphorimaging screens.

In conclusion, IntN1 shows a preference for the bottom strand of the attN1 site. Following conjugation, ApR, KnR, and CmR colonies were obtained only if the attN1 plasmid integrated into the pir recipient chromosome (Fig. 2). A donor strain carrying the pEPE plasmid was used as the negative control. The pEPE plasmid is the parent vector of pEPE:attN1 that lacks the attN1 site. In the absence of an attN1 site, the integration frequency was <10^-7 integrants per recipient. We tested two donor strains, each car-
that anneal to the att sites such that amplification of attN1, attBT1-1, attL, and attR gave different sized fragments (3, 13). In >90% of the colonies the attL and attR sites were obtained by site-specific recombination. When the parental plasmid pEPE (lacking attN1 site) was electroporated into the recipient cells, no Ap-, Kn-, and Cm-resistant colonies were obtained. This demonstrates that IntN1 is able to use a double-stranded circular substrate in vivo. The similar experiment performed with integron cassettes revealed that the integron integrase IntI1 could not act on a transformed double-stranded attC plasmid (17).

A caveat is that conjugation in E. coli with recombinant plasmids may not be equivalent to natural transfer of NBU1 between Bacteroides species. Although we cannot rule out the possibility that IntN1 is able to process both single- and double-stranded forms of attN1, or that the active target is formed by either the top or the bottom strands of attN1, the most compelling evidence for the use of a double-stranded substrate comes from the in vitro system that uses both double-stranded substrates (see next section).

In Vitro Integration Assay for IntN1—Previous analysis of NBU1 site-specific recombination utilized an in vivo conjugation assay. To facilitate mechanistic studies and to avoid processing of intermediates in vivo by host enzymes, we developed an in vitro recombination assay. In this system, NBU1 IntN1 and a host factor were added to reaction mixtures containing a supercoiled plasmid pJWS200 carrying the NBU1 attN1 site, and a short linear radiolabeled fragment (44 bp) containing the attBT1-1 site. The in vitro reactions were set up with either WT attBT1-1 (filled squares) or the mutant C(−3)G attBT1-1 (open triangles). Aliquots were removed at 10, 30, 60, 120, 180, 240, 300, and 360 min and the reactions were stopped by addition of 1% SDS. Recombination percentage was calculated as described under “Experimental Procedures.” The graph is representative of at least three independent experiments.
kb in size. The recombination scheme and the expected restriction products are shown in Fig. 3A, and the results (Fig. 3C) are consistent with the product being formed by site-specific recombination between the attN1 and attBT1-1 sites.

The optimal recombination conditions were at a temperature of 37°C, a pH of 8, and in the presence of dithiothreitol and spermidine. Dithiothreitol was essential for the reaction to occur. We found that increasing the glycerol concentration to 40–50% could substitute for spermidine. The reaction was not stimulated by the addition of Mg2+, ATP or Me2SO. We also tested if supercoiling of the attN1 plasmid was necessary. When the attN1 plasmid was relaxed using a topoisomerase or linearized by Scal digestion, no product formation was seen (not shown).

We tested various cell extracts and nucleoid proteins to see if they stimulate the reaction. A host factor is essential for the reaction. The reaction was stimulated greatly either by a partially purified specific Bacteroides host factor3 or purified E. coli IHF. On the other hand, when the reaction was supplemented with either a Bacteroides crude extract or purified HU protein, the amount of product formed was low compared with when IHF was added. Integration of CTnDOT in vitro is also stimulated by E. coli IHF, but not by HU (19). We used the E. coli IHF as the host factor in all further experiments described here.

One of the attBT1-1 crossover mutants that was previously constructed, C(−3)G attBT1-1, enhanced in vivo integration frequencies by 100-fold (13). The C(−3)G attN1, however, was deficient in recombination. A cross between C(−3)G attBT1-1 and C(−3)G attN1, where the sites are homologous, had an integration frequency less than that between two wild-type sites (Fig. 1B). We tested the C(−3)G attBT1-1 in the in vitro reaction against wild-type attN1. We observed that the mutant site had a much higher rate of reaction than the wild-type site (Fig. 4A). We observed that the reaction with the top strand nicked substrate resulted in the accumulation of an intermediate that migrated slower on an agarose gel than the linear recombinant (Fig. 4A, lane 2). Only a minor amount of product that migrated like the linear recombinant was obtained with the bottom strand nicked attBT1-1 (Fig. 4A, lane 5). This difference in the reaction products between the two nicked substrates implies that there is a defined order of strand exchanges. It is likely that NBU1 integration proceeds by exchange of bottom strands first. When the nick was at the top strand cleavage site, IntN1 could cleave and exchange the bottom strands, but top strand exchange could not occur and the reaction intermediate accumulated. Such a Holliday junction intermediate was expected to migrate similar to the relaxed pJWS200 plasmid due to the presence of the nick. The mobility of this band did not change upon treatment with proteinase K.

The minor amount of product seen with the substrate having a nick on the bottom strand cleavage site may be due to a small percentage of substrates carrying a 5′-OH at the nick instead of a 5′-PO4, which could then carry out strand transfer of the bottom strand of attN1 to the 5′-OH of the nicked attBT1-1 bottom strand. Although not visible in Fig. 4A, a similar band was also seen with the top strand nicked attBT1-1. Such asymmetric strand transfers that gave rise to linear recombinants were observed with λ Int using nicked suicide substrates carrying a 5′-OH at the nick (18).

To ensure that the different results seen were entirely due to the position of the nick alone, and not because the substrates were not formed properly after annealing, both the substrates were incubated with T4 DNA ligase to seal the nick, and then used in the in vitro recombination reaction. Both the top strand and the bottom strand substrates, when treated with T4 DNA ligase, generated the linear recombinant indicating that the original substrates contained the nicks (Fig. 4A, lanes 3 and 6).

Restriction Analysis of the Holliday Junction Intermediate—As shown previously in Fig. 3C, restriction digestion of the linear recombinant with Scal produced two fragments, ~1.4 and 2.1 kb in size. Scal digestion of an alpha structure Holliday junction intermediate should result in only one product with an X shape. Because the X-shaped product would have two plasmid arms (~1.4 and 2.1 kb) much longer than the two substrate arms (~20 bp each), it should be similar in electrophoretic mobility to the linear recombinant. Scal digestion of the Holliday junction intermediate resulted in a single band that migrated at an approximate size of 3–4 kb (Fig. 4B, lane 2). This further supports the hypothesis that the recombination intermediate obtained with the top strand nicked substrate is an alpha structure-shaped Holliday junction.

Denaturing Gel Analysis of the Reaction Products—To determine if the reaction intermediate obtained with the substrate carrying a nick at the site of top strand cleavage is a Holliday junction intermediate, the reaction products were electrophoresed on a denaturing alkaline-agarose gel. The scheme shown in Fig. 4C depicts the predicted reaction products. The linear recombinant that is obtained using a radiolabeled attBT1-1 with no nick, when denatured, would give long labeled single strands. If the reaction intermediate obtained with the nicked substrate is an α structure Holliday junction

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3 M. Romero-Guss, unpublished results.
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Intermediate with only the bottom strands exchanged, then the radiolabeled denatured products would differ depending on which strand of the substrate is labeled. If the top strand is labeled, then the only labeled product expected after denaturation will be the short substrate strand. On the other hand, if the substrate is labeled on the bottom strand, denaturation should result in a long exchanged bottom strand that is labeled, the same size as the single strands obtained from the intact attBT1-1.

The results obtained as shown in Fig. 4C were consistent with the predicted scheme. In reactions with intact attBT1-1 (Fig. 4C, lanes 1 and 2), the long labeled single strands were obtained. The same product was seen with the nicked substrate only when it was labeled on the bottom strand. When the label was placed on the top strand, the entire label stayed with the substrate (Fig. 4C, lanes 3 versus 4). This supports our hypothesis that the reaction intermediate that accumulated was a Holliday junction intermediate with only the bottom strands exchanged.

**Use of Peptides That Trap Holliday Junction Intermediates**—By screening synthetic peptide combinatorial libraries, peptides have been identified that trap Holliday junction intermediates in phage λ recombination reactions and prevent their resolution (22–24). These peptides also inhibit recombination by other tyrosine recombinases such as Cre, Flp, XerC, and XerD (24–26). The use of peptides enables the observation of reaction intermediates using natural substrates. We used a dodecameric peptide (WRWYRGGRYWRW) in the NBU1 in vitro integration assay. This peptide, unlike many of the other well-characterized peptides, is not reduced by dithiothreitol, and this allowed us to use it in the NBU1 in vitro reaction.

We found that the peptide significantly inhibited the IntN1 recombination at a final concentration of 50 µM and trapped an intermediate that migrated like the supercoiled attN1 plasmid (Fig. 5A, lane 3). Base-pairing within the arms would constrain the circular molecule, and therefore most of the α structure is expected to be supercoiled (21). A trace amount of intermediate can also be observed in reactions lacking the peptide (Fig. 5A, lanes 1 and 2). With lower amounts of the peptide, the recombination was not inhibited greatly, and only a small amount of intermediate was trapped. This peptide blocked λ Int pathways with a 50-fold higher potency.4 This was expected, because the peptide was developed with λ Int and substrates as targets.

ScaI digestion of the intermediate was consistent with it being an alpha structure Holliday junction intermediate (not shown). The reaction with the peptide also accumulated a smaller amount of an additional band that migrated much slower than the linear recombinant (Fig. 5A, lane 3; Fig. 5B, lanes 6 and 7). We believe that this is likely the Holliday junction intermediate that is nicked and migrates as a relaxed plasmid. It is possible that some relaxation occurs by topoisomerase

4 J. L. Boldt and A. Segall, unpublished observation.
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FIGURE 5. Dodecamer peptide accumulates recombination intermediate in the NBU1 in vitro integration reaction. S, substrate; R, linear recombinant; HJ, Holliday junction; N-HJ, nicked Holliday junction; 2x and 3x, multimers obtained by second and third rounds, respectively, of recombination between attN1 and one of the recombinant sites. A, peptide inhibits recombination at 50 μM concentration. Recombination reactions were set up as described previously. Lane 1, no peptide; lane 2, 0.5% Me₃SO added (control reaction to check effect of Me₃SO on the reaction, because the peptide stock solution was in Me₃SO); lanes 3–6, reactions with peptide at 50, 25, 12.5, and 6.25 μM concentrations, respectively. B, effect of peptide on WT versus C(-3)G attBT1-1. Reactions were set up as with WT attBT1-1 (lanes 1–4) or C(-3)G attBT1-1 (lanes 5–8), and the reactions were supplemented with peptide: lanes 1 and 5, no peptide; lanes 2 and 6, 62 μM; lanes 3 and 7, 50 μM; and lanes 4 and 8, 31 μM peptide. C and D, two-dimensional agarose gel analysis of the recombination intermediate. In vitro recombination reactions were supplemented with the peptide, and incubated for 2 h. The reactions were run on a native agarose gel in the first dimension, and then under denaturing conditions in the second dimension. C, C(-3)G attBT1-1 labeled on the top strand was used. D, C(-3)G attBT1-1 labeled on the bottom strand was used.

activity of IntN1 at its second cleavage site in attN1. Although we have not observed generation of topoisomers by IntN1, incubation of IntN1 with a supercoiled attN1 plasmid results in a small increase in the amount of the nicked circular form. The supercoiled and relaxed forms of the Holliday junction were also observed in λ Int reactions that were blocked by a non-bridging phosphorothioate at the site of second strand exchange (21).

To determine if the Holliday junction intermediate trapped by the peptide also has the bottom strands preferentially exchanged, we first separated the reaction components on a native agarose gel. The single strand components of each fragment were then separated by running a second dimension under denaturing conditions. When the attBT1-1 was labeled on the top strand (Fig. 5C), the denatured Holliday junction migrated similar to the labeled attBT1-1 indicating that the top strands were not exchanged. On the other hand, when the attBT1-1 was labeled on the bottom strand, the denatured Holliday junction migrated like the linear recombinant indicating that the bottom strands were exchanged (Fig. 5D). Thus, the intermediate obtained by both the peptide and the nicked attBT1-1 substrates had the bottom strands exchanged.

We also tested the peptide in a reaction with the wild-type attBT1-1. Surprisingly, the peptide did not inhibit the recombination reaction with wild-type attBT1-1, and we did not observe a faster migrating intermediate (Fig. 5B). However, a minor amount of a band that migrated similar to the nicked Holliday junction seen with C(-3)G attBT1-1 was present in the reactions of wild-type attBT1-1 with peptide. We do not know if this band represents a small amount of Holliday junction intermediate that was trapped, but in a conformation different from that predominately seen with the mutant attBT1-1. It is clear, however, that the peptide has different potencies with the wild-type versus the C(-3)G attBT1-1 substrates. Analysis of the various recombination pathways of λ Int showed that the more efficient a pathway, the more potent the peptide was at trapping junctions (24). Although the reaction with the wild-type substrate is much less efficient, we did not observe any inhibition of the wild-type recombination with the peptide.

Effect of Crossover Mismatches on the in Vitro Integration Reaction—Because the attBT1-1 substrate with the C(-3)G substitution had a much faster rate of reaction and higher efficiency than the WT attBT1-1, we were interested in determining the effect of other substitutions at this position. We tested two attBT1-1 substrates carrying the substitutions C(-3)A and C(-3)T in reactions with wild-type attN1. Both these substrates were deficient in the reaction (not shown). Only the C(-3)G attBT1-1 had the stimulatory effect.

We also tested supercoiled plasmids carrying the attN1 site with the G(-2)C or the C(-3)G substitution for recombination with the WT attBT1-1. Consistent with the in vivo results, the G(-2)C attN1 had a higher rate of reaction than the WT attN1, whereas the C(-3)G attN1 gave no product (not shown).

Because the G(-2)C attN1 and the C(-3)G attBT1-1 were able to independently enhance the integration reaction, we tested if the recombination reaction between the two mutant sites would be stimulated further. However, no product was formed with this cross (not shown).

To determine the effect of mismatches on the left side of the crossover region, we tested the A(-8)T and G(-7)C attBT1-1 substrates in the in vitro integration assay. We observed that the amount of linear recombinant obtained with A(-8)T or G(-7)C was less than that seen with the WT attBT1-1. Instead, recombination intermediates accumulated (Fig. 6). Two bands were observed in addition to the recombinant band, one that migrated faster and presumably is the supercoiled Holliday junction intermediate, and the second that migrated slower than the recombinant and is the relaxed Holliday junction intermediate. These reaction intermediates are thus similar to those that were trapped by the peptide with the C(-3)G attBT1-1 substrate. The Holliday junction is most likely formed by the exchange of bottom strands, but the reaction could not be completed probably, because base-pairing and ligation for the second strand exchange could not occur in the presence of
mismatches. It is to be noted that a small percentage of the reaction appeared to proceed to completion, suggesting that IntN1 was able to ligate some of the mispaired strands and resolve the Holliday junction despite the mismatch. However, substitutions at positions A(−8) or G(−7) did not significantly affect the in vivo integration frequency (13). It is likely that in vivo the Holliday junction is resolved by cellular processes such that wild-type integration levels are observed.

DISCUSSION

In Vitro System for NBU1 Integration—We have developed here an in vitro system that utilizes a supercoiled attN1 plasmid and a linear attBT1-1 to generate linear recombinants in the presence of IntN1 and E. coli IHF. This is the first report of an in vitro system for integration of a mobilizable transposon that uses a tyrosine recombinease.

With the recent discovery that folded single-stranded DNA can form substrates for certain tyrosine recombinases (16, 17), it was proposed that similar mechanisms could be employed by conjugative and mobilizable transposons where the single-stranded form is part of the natural life cycle. However, we were unable to derive any obvious secondary structures from the attN1 sequence that could fold into an appropriate target. In this report we showed that NBU1 integration in vivo proceeded with the same efficiency when either the top or the bottom strand of attN1 entered the recipient cell. We also observed that double-stranded attN1 plasmids could be transformed into E. coli-recipient cells and integrated into the chromosomal attBT1-1 site. Because the in vitro system utilizes both double-stranded substrates, we believe the most likely scenario for NBU1 transfer is that, following single strand transfer into the recipient cell, the complementary strand is synthesized, and IntN1 acts on the double-stranded substrate resulting in NBU1 integration.

Order of Strand Exchanges—Does IntN1 carry out ordered strand exchanges via a Holliday junction intermediate? To answer this question, we used the in vitro integration system to trap reaction intermediates.

We used attBT1-1(C(−3)G) substrates that are nicked either at the top or the bottom strand cleavage site. We found that a nick at the bottom strand cleavage site completely blocked most of the reaction, whereas a nick at the top strand site formed an intermediate, which we showed by denaturing gel electrophoresis and restriction digestion to be the α-structure Holliday junction intermediate having the bottom strands exchanged. The same bias was observed when nicked substrates with a wild-type crossover region were used (not shown). IntN1 seems to follow a strict order of strand exchanges (Fig. 4). This is also true for λ Int (18, 27), XerCD (28), as well as for CTnDOT Int.5 The Holliday junction intermediate was also accumulated with intact attBT1-1 substrates using a dodecameric peptide, and it was also formed by the same preferential exchange of bottom strands (Fig. 5). Thus, we have shown using two independent methods that IntN1 integration proceeds via a Holliday junction intermediate that is formed by the exchange of bottom strands.

Effect of Mismatches on Strand Exchanges—The requirement for homologous crossover sites in tyrosine recombinase reactions has been well studied for λ Int, Cre, and Flp (5–10, 18, 21). In λ Int in vitro reactions, a mismatch at any of the 7 bp within the overlap region reduced recombination drastically (21). Recombination between homologous mutant att sites proceeded with efficiencies close to or slightly lower than wild type (5, 21). When the heterology was located to the right of the overlap region, Holliday junction intermediates were seen, whereas no product was formed when the heterology was on the left side (18, 21). An extensive mutational analysis has been performed on the Cre loxP sites (10). Single base pair substitutions in any of the 6 bases within the spacer inhibited recombination. Mismatches at the two positions on the right side of the spacer resulted in very little intermediate or complete recombinant, whereas mismatches in the 4 bases on the left side accumulated reaction intermediates (10). It appears that heterologies near the first strand exchange site inhibit formation of intermediates, whereas mismatches near the second strand exchange site accumulate intermediates and prevent formation of the recombinant.

In stark contrast to the above mentioned systems, NBU1 integration is stimulated by heterology at two positions next to the first strand exchange site. We demonstrated in this report that, consistent with the in vivo integration assays (13), the C(−3)G attBT1-1 substrate is more efficient than the wild type in the in vitro system. We also showed that recombination with C(−3)G attBT1-1 does occur via a Holliday junction formation. Because recombination occurs by the cleavage and exchange of bottom strands first, this would involve the exchange of bases −2 to −4 (Fig. 1) thus creating mismatches between the NBU1 att sites. For Flp (12) and λ Int (29), it was shown using in vitro assays with half and full sites that the ligation step is sensitive to homology, and efficient ligation requires base-pairing between the two or three bases that are swapped. IntN1 is different from these systems, because a mismatch at position −2 (G(−2)C in attN1) or at position −3 (C(−3)G in attBT1-1) not only allows the reaction to occur but increases its efficiency. The strand-swapping model that requires base-pairing (11, 12) cannot account for these results. IntN1 therefore must be able to carry out strand ligation when the first or the second base next to the strand exchange point is mispaired.

Holliday junction intermediates were accumulated when mismatches were introduced at the second strand exchange site, as seen in in vitro reactions between wild-type attN1 and

5 K. Malanowska, S. Yoneji, A. A. Salyers, J. F. Gardner (2007) Nucleic Acids Res., in press.
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G(−7)C or A(−8)T _attBT1-1_. Similar results were obtained for λ Int and Cre with heterologies near the second strand exchange site. In contrast to these systems, where no complete recombinants were observed, NBU1 IntN1 formed a small amount of linear recombinant (Fig. 6). This indicates that IntN1 is able to ligate some of the mispaired strands. It appears that IntN1 can tolerate heterology at the second strand exchange site better than λ Int and Cre. However, IntN1 is different from the _Bacteroides_ CToDOT integrase, which is insensitive to any heterology at the second strand exchange site and can efficiently ligate mispaired substrates.\(^6\)

*Analysis of the Mutant Sites That Increase Recombination Frequency—How do the C(−3)G _attBT1-1_ and G(−2)C _attN1_ substitutions make the _att_ sites better substrates? Recombination between homologous mutant _att_ sites is less efficient than that between two wild-type sites. Therefore, DNA homology is not playing a role in reactions with these substrates. Because the stimulatory effect of the G(−2)C substitution is seen only in _attN1_, and that of C(−3)G only in _attBT1-1_, and because the sequences immediately to the right of the crossover region are different in the two _att_ sites, we had previously proposed that these substituted bases make protein-DNA contacts in the intasome that highly favors the reaction (3). The substituted bases could be increasing the binding affinity of the intasome to _attN1_, or the intasome is better able to capture the C(−3)G _attBT1-1_. Alternatively, the substitutions could be increasing the strand cleavage or ligation efficiency. However, cleavage of half site _attBT1-1_ and _attN1_ substrates was not affected by the substitutions.\(^6\)

Another possibility is that the substituted bases favor the formation or the resolution of the Holliday junction intermediate. Reactions with the peptide suggest that the Holliday junction formed with the C(−3)G _attBT1-1_ may adopt a different structure than that with the wild-type site. The peptide did not inhibit the reaction with the wild-type substrate, whereas it reduced the amount of completed recombinants formed and accumulated Holliday junction intermediates with C(−3)G _attBT1-1_. The presence of the heterology in the C(−3)G Holliday junction may alter its structure such that the peptide can now efficiently trap it. The inhibitory activity of the various peptides tested varies significantly between the integrative, excisive, bent-L, and straight-L pathways for λ Int recombination (24). This is presumed to be because of subtle differences in the conformations of the Holliday junction intermediates made in these pathways. However, when the peptide WKHNYW was tested in λ Int bent-L and excisive pathways with _saF_ mutants, the heterology did not significantly affect the amount of Holliday junction intermediates trapped as compared with the efficiency of the recombination (23). Perhaps it is not the heterology alone that changes the conformation of the IntN1-Holliday junction, but the favorable contacts formed in the intasome by the substituted base.

It is interesting to note that the reaction between G(−2)C _attN1_ and C(−3)G _attBT1-1_ yielded no products, although each substrate is very efficient in reactions with the wild-type partner. It is possible that the presence of two adjacent mismatches overcomes any synergistic effect that might have occurred.

**CONCLUSION**

IntN1 utilizes a mechanism involving ordered sequential strand exchanges and a Holliday junction intermediate. However, the first pair of strand exchanges shows apparent homology independence. In this respect, IntN1 differs from the tyrosine recombinases that demonstrate a strict dependence on homology within the entire crossover region such as λ Int, Flp, and Cre and also from the tyrosine recombinase IntN1, only one of the first strand exchange appears to be homology-dependent. IntN1 expands the diversity of tyrosine recombinases by being the only known recombinase whose efficiency is drastically increased by particular mismatches within the overlap region. The sub-optimal sequence of the NBU1 crossover region probably exists to preserve the integrity of the _leu-tRNA_ gene into which it inserts.

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