Multiple DNA Binding Activities of the Novel Site-specific Recombinase, Piv, from *Moraxella lacunata* *

(Received for publication, August 5, 1998, and in revised form, January 20, 1999)

Deborah M. Tobiason, Anne G. Lenich, and Anna C. Glasgow‡

From the Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322

The recombinase, Piv, is essential for site-specific DNA inversion of the type IV pilin DNA segment in *Moraxella lacunata* and *Moraxella bovis*. Piv shows significant homology with the transposases of the IS110/IS492 family of insertion elements, but, surprisingly, Piv contains none of the conserved amino acid motifs of the λ Int or Hin/Res families of site-specific recombinases. Therefore, Piv may mediate site-specific recombination by a novel mechanism. To begin to determine how Piv may assemble a synaptic nucleoprotein structure for DNA cleavage and strand exchange, we have characterized the interaction of Piv with the DNA inversion region of *M. lacunata*. Gel shift and nuclease/chemical protection assays, competition and dissociation rate analyses, and cooperative studies indicate that Piv binds two distinct recognition sequences. One recognition sequence, found at multiple sites within and outside of the invertible segment, is bound by Piv promoters with high affinity. The second recognition sequence is located at the recombination cross-over sites at the ends of the invertible element; Piv interacts with this sequence as an oligomer with apparent low affinity. A model is proposed for the role of the different Piv binding sites of the *M. lacunata* inversion region in the formation of an active synaptosome.

Numerous site-specific DNA recombination systems and DNA transposition systems have been characterized biochemically and have been found to follow two distinct chemical pathways for DNA cleavage and strand transfer in recombination (reviewed in Refs. 1–4). Site-specific recombination, mediated by the recombinases of the λ-integrase and Hin/resolvase families, involves a two-step transesterification reaction in which the intermediate is a covalent recombinase-DNA linkage. This covalent attachment is the result of nucleophiliic attack on the DNA phosphodiester backbone by a hydroxyl group of the conserved serine (Hin/resolvase), or tyrosine (λ-integrase), of the recombinase. In the second transesterification reaction, the phosphodiester linkages of the exchanged DNA strands are restored (reviewed in Refs. 2 and 3). In contrast, DNA transposition, mediated by transposases containing the catalytic DDE amino acid motif, utilizes a hydrolysis reaction for cleavage at the ends of the transposable element. This first cleavage leaves 3′-OH ends to act directly as the attacking nucleophile in a one-step trans-esterification reaction resulting in strand exchange. Resolution of the transposition process involves DNA replication or DNA repair activity to fill in gaps left at the target site due to the staggered cut mediated by the transposase and the 3′ hydroxyl groups at the element ends (reviewed in Refs. 1 and 4).

These features of the recombination reactions mediated by site-specific recombinases and transposases suggest that a group of related recombinases would not mediate both site-specific recombination and transposition. Therefore, it is surprising that the site-specific recombinase Piv, which directs site-specific DNA inversion in *Moraxella lacunata* and *Moraxella bovis*, exhibits significant homology to the transposases of the IS110/IS492 family of IS elements (approximately 25–35% amino acid identity and 45–55% similarity, 5). Furthermore, Piv and the IS110/IS492 transposases do not appear to be related to the site-specific recombinases of the λ-Int or Hin/resolvase families or the transposases containing the DDE motif (4). Therefore, Piv and the IS110/IS492 transposases may define a new family of DNA recombinases.

The homology between Piv and the IS110/IS492 transposases includes several highly conserved amino acid regions (5), which, based on mutational analyses, contain functionally relevant amino acid motifs. 3 Although there is no completely conserved DNA sequence among all the IS elements and the Piv invertible DNA segment, there is a consensus sequence for the ends of a subgroup of the IS elements, which is also found overlapping the recombination sites of the Piv invertible element. 3 To determine if indeed Piv and the IS110/IS492 transposases define a new family of DNA recombinases that utilize a common mechanism for both site-specific recombination and transposition, we must characterize the recombination reactions mediated by Piv and transposases from the IS110/IS492 family. As a first step in understanding the mechanism for Piv-mediated assembly of a synaptic nucleoprotein complex, DNA cleavage, and strand exchange, we have characterized the interactions of Piv with the invertible DNA segment of *Moraxella lacunata*.

**MATERIALS AND METHODS**

Reagents—DNase I was obtained from Worthington Biochemical Corp. Amylose resin, restriction enzymes, T4 DNA ligase, Klenow (exo−), and T4 polynucleotide kinase were purchased from New England Biolabs (NEB). *Pfu* DNA polymerase was obtained from Stratagene. The His tag XPRESS purification system and the TA cloning kit were purchased from Invitrogen. Radionucleotides were obtained from NEN Life Science Products. Isopropyl-1-β-D-galactopyranoside (IPTG), 2

* This work was supported by Public Health Service Grant GM49794-05 from the National Institutes of Health, by American Cancer Society Junior Faculty Research Award JFRA-501 (to A. C. G.), and by Presidential Young Investigator Award MCB-9396003 from the National Science Foundation (to A. C. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Microbiology and Immunology, Emory University School of Medicine, 1510 Clifton Rd., Atlanta, GA 30322. Tel.: 404-727-3734; Fax: 404-727-3659; E-mail: acglasg@bimcore.emory.edu.

‡ This paper is available on line at http://www.jbc.org
dithiothreitol, and EDTA were purchased from Sigma. Dimethyl sulfate was purchased from Aldrich.

**Plasmids**—The pAG607 plasmid is composed of the pMal-C2 vector (NEB) containing the pvi gene inserted into the XmaI and BamHI sites such that it is in frame directly downstream of a factor X protease site in the pMAL-C2 vector. The pvi gene was amplified by polymerase chain reaction (PCR) from the pMalXI plasmid, subclone of M. lacunata inverted region provided by C. Marris (6), using Pfu DNA polymerase and the primers: 5’-GCCAGCAAGTGTCTAATACATCATTGAT-3’ and 5’-CTAATGCTTTGAGGAGAATT-3’. Similarly, the pvi gene from pMxl was PCR-amplified with the primers: 5’-CTGGTGCTCGTTCGATCTCGT-3’ and 5’-CTCGAATGCGTTAAACCTTACACTGTTAC-3’. This 245-bp fragment, containing the strong upstream binding site (sub1), was cloned into the pCR2.1 vector (TA cloning kit, Invitrogen). The resulting plasmids, pAG604 and pAG605, contain sub1 in opposite orientations. The inserted DNA was sequenced (T7 Sequenase version 2.0 DNA sequencing kit; Amersham Pharmacia Biotech) to check for any mutations due to the PCR reaction.

To construct the DNA inversion test plasmid pAG686, a 5865-bp DNA fragment containing the invertible segment was obtained by partial digestion of pMxl with EcoRI. This fragment was gel-purified and ligated into EcoRI-digested pACYC184 (NEB) creating the pAG850 plasmid. To inactivate pvi, the Ω fragment encoding the SpeI/SmaI genes was recombined into the pvi gene of pAG850 by homologous recombination with pMAL5 plasmid (6) in the BglII site of pT7-1 and pMAL (NEB). The recombinant plasmid, pAG862, was confirmed by restriction digestion and DNA sequencing.

**Protein Purification**—To produce the Piv protein fused at its amino terminus to maltose-binding protein (MBP-Piv), DH5α containing pAG607 was grown in Luria broth to OD_{600}=0.5, induced with 0.5 mM IPTG, aerated at 37 °C for 2 h, and lysed in a French pressure cell. Crude extract was immediately loaded onto an anion exchange column, washed with column buffer (20 mM Tris-Cl, pH 7.4, 500 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol), and then eluted with 10 mM maltose in column buffer (7, 8). Fractions were collected and frozen in 20% or 30% glycerol at −20 °C. Protein concentration was quantitated using the Bio-Rad DC protein assay kit.

The His-tagged Piv, pAG300 was introduced into BL21(DE3) (Novagen), grown to mid-log phase in Luria broth, then induced with 1 mM IPTG. After 1 h aeration at 37 °C, cells were collected by centrifugation, resuspended in 20 mM phosphate, 500 mM NaCl, pH 7.8, and lysed in a French pressure cell at 4 °C. The lysate was centrifuged at 30,000 × g. The supernatant was loaded onto an Invitrogen ProBond column (Ni²⁺ affinity column). Following washes with 20 mM phosphate, pH 7.5 buffer, protein was eluted with an imidazole step gradient (50, 200, 350, and 500 mM). Piv-His₉ eluted at 300 mM imidazole. The protein was dialyzed into 20 mM phosphate, 500 mM NaCl, pH 7.8, and 30% glycerol at 4 °C and stored at −20 °C.

**Antibody Production and Purification**—Antibody to the Piv protein was produced in rabbits injected with purified MBP-Piv protein (BabCQ; Berkeley Antibody Company). Nonspecific antibodies were removed from the rabbit sera by absorption to whole cells of DH5α cultures, followed by multiple passages over a Sepharose column bound with Escherichia coli proteins and MBP. This affinity column was made by lysing a culture of pMal-C2/DH5α that had been induced with 0.5 mM IPTG to express the MBP protein, then binding the proteins from this lysate to cyanogen bromide-activated Sepharose resin using the protocol of the resin manufacturer (Amersham Pharmacia Biotech). The eluate was stored at 4 °C.

**Western Blot and SDS-PAGE Analysis**—SDS-polyacrylamide gel electrophoresis was performed using the protocol of Sambrook et al. (10) with the following modifications; polyvinylidene difluoride (PVDF) nitrocellulose membranes (Bio-Rad) were used for blotting, and the blot was probed with a 1:400 dilution of partially purified Piv antibody. The binding buffer was an SDS-PAGE standard, low range, or Kaleido-scope prestained standards (for Western blots) from Bio-Rad.

**Inversion Assay**—DH5α containing pAG862, pAG862 and pAG607 or containing pAG862 and pMal-C2 were grown to OD_{600} = 0.6, induced with 0.05 mM IPTG, and incubated for 2 h at 37 °C. Plasmids were extracted using an alkaline lysis protocol (9) and digested with HindIII and KpnI. The restriction digests were examined on a 0.8% SeaKem (FMC Bioproducts) agarose gel. The DNA was visualized by ethidium bromide staining and documented by digital imaging of the UV-illuminated gel.

**Protocol of DNA Fragments Used in DNA Binding Assays**—Double-stranded DNA oligonucleotides, INV and SUB (see “Results”), were labeled with [γ-32P]ATP using T4 polynucleotide kinase following the protocols of Sambrook et al. (9). pAG604 and pAG605 were digested with SpeI and EcoRV to obtain 298-bp fragments containing the sub1 site to be used in DNA I protection assays. These fragments were labeled with [γ-32P]ATP using Klenow (exo⁻) DNA polymerase under conditions recommended by the manufacturer with the modification of incubating the reactions at 25 °C; the labeled fragments were electrophoresed on a 5% polyacrylamide gel, electroeluted from the gel, and stored at −20 °C. The pmxlI-dl24 plasmid, a deletion derivative of pmxlI, provided by C. Marris (6), and the pmxlI invR fragment (obtained by PCR from pmxlI, using Pfu DNA polymerase and the primers: 5’-GCCAGCACGTCTTAACACTTACG and 5’-CCCATACCTAGCACAGCCAGC) were digested with various restriction enzymes as indicated under “Results.” The DNA fragments created were labeled with [α-32P]ATP or dCTP using Klenow (exo⁻) polymerase, gel-purified, and stored at −20 °C.

**Gel Electrophoresis Retardation Assays**—Gel electrophoresis retardation assay was performed by mixing of DNA with each radiolabeled DNA fragment (5 × 10^{-11} M) in 1× binding buffer: 80 mM KCl, 20 mM Tris-Cl, pH 7.6, 5 mM CaCl₂, 250 mM poly(υC), 1 mM dithiothreitol, and 50 mg/ml bovine serum albumin (NEB). The reactions were incubated for 20 min at room temperature, immediately loaded onto a nondenaturing polyacrylamide gel, and run at 4 °C in a 5× TBE buffer. For the competition assays, increasing amounts of competitor DNA ranging from 1 to 500 molar excess was added to the reaction before addition of the protein. The specific DNA competitors were the SUB and INV double-stranded oligonucleotides and the nonspecific competitor DNA was a 39-bp oligonucleotide: 5’T-GTAATCGTGATCGCTGATCGCATCGTAAGCG-3’.

**Nuclease and Chemical Cleavage Assays**—For the DNA I protection assays performed at the sub1 site, the 298-bp fragments from pAG604 and pAG605, labeled on the top strand and bottom strand for sub1 (5 × 10^{-11} M), were incubated for 20 min at room temperature with or without MBP-Piv (7 × 10^{-10} M) in 1× binding buffer. DNA I protection assays were performed in binding buffer plus 5 mM MgCl₂ with 0.02 units of DNA I as described previously (11). Chemical cleavage using MBP-Piv was performed in binding buffer plus 5 mM MgCl₂ with the same labeled fragments described above. The footprinting and sequencing reactions were run on a 6% sequencing polyacrylamide gel. At the site of inversion, dimethyl sulfate interference assays were performed as described previously (11). Indirect DNA I and 1,10-phenanthroline-copper protection assays were also performed at the site of inversion following the protocol described by Kuwabara and Sambrook (11). These modifications for the DNA I protection assay: gel slices were treated with DNA I in 1× binding buffer plus 5 mM MgCl₂ for 5–8 min before quenching the reaction with the addition of 20 mM EDTA.

**Cooperativity Studies**—Gel electrophoresis retardation assays were performed with labeled DNA fragments (5 × 10^{-12} M) and increasing amounts of MBP-Piv protein ranging from 0.6 to 1552 mM for binding of SUB and 0.6 to 4560 mM for binding to INV. Binding of MBP-Piv (P) to each DNA binding site (X) is represented by the equation: K = [P·X]/[P·X], which translates into ln[P·X/X] = n ln[P] − ln(K). [P] is the concentration of MBP-Piv monomers, [Pₙ] is the concentration of active MBP-Piv oligomers, n is the number of active monomers in the oligomer, [P·X] is the concentration of bound DNA, and [X] is the concentration of unbound DNA. A plot of ln[P·X/X] versus ln[P·X] gives a line with slope n (14). The amount of bound and unbound DNA was quantitated using a PhosphorImager: 445SI with ImageQuant software (Molecular Dynamics).

**Dissociation Rate Analysis**—Binding assays were performed in 1× binding buffer. Labeled, double-stranded oligonucleotide (5 × 10^{-10} M) was incubated with MBP-Piv protein (6 × 10^{-7} M) as indicated, for 20 h at 20 °C. Following incubation, the samples were loaded onto a 10% nondenaturing polyacrylamide gel running at 120 V. Following electrophoresis, the gels were dried and imaged with a PhosphorImager: 445SI. ImageQuant software was used to quantitate the label in the bands corresponding to MBP-Piv-DNA complexes and unbound DNA. The fraction of the total DNA in the reaction that was bound was calculated from the ratio of band intensity to unbound DNA intensity.
with 0.5 mM IPTG, containing pMal-C2 (MBP) or pAG607 (MBP-Piv), soluble and expressed at high levels. Affinity chromatography produce the fusion protein, MBP-Piv. MBP-Piv (79 kDa) was with Coomassie Blue, showing 20

m

predicted to be a 322-amino acid polypeptide with a

M

of the proteins expressed from cultures, uninduced (U

B

) or induced (I

B

) with 0.5 mM IPTG and 20

m

con-
DNA inversion test plasmid, pAG862, and expression plasmids encoding either MBP alone (pMal-C2), or MBP-Piv (pAG607). After overnight expression of MBP or MBP-Piv, recombination of the invertible segment on pAG862 was assayed by restriction enzyme digestion of isolated plasmid DNA. In the original orientation of the test plasmid, digestion with HindIII and KpnI results in two bands, of 5.7 and 1.0 kb. If there is MBP-Piv-mediated inversion, the same digestion will yield two different unique bands, of 4.1 and 2.6 kb. With MBP alone, no inversion products are seen, whereas, in the presence of MBP-Piv, approximately 50% of the test plasmid is in the inverted orientation (Fig. 2). In a similar inversion assay, wild type Piv also directed inversion to yield approximately 50% of either orientation, representing an equilibrium point in the inversion reaction (data not shown). Thus, these results indicated that the MBP-Piv fusion is a functional recombinase; consequently, the uncleaved MBP-Piv fusion protein was used in subsequent in vitro DNA binding studies.

Piv Binding Sites within the Inversion Region of M. lacunata—Based on the binding specificity of other site-specific recombinases and transposases (reviewed in Refs. 1 and 2), we expected that Piv would bind DNA sequences encoding the cross-over sites for inversion (invL and invR) as well as possible accessory sites involved in recombination or regulation of piv expression. An assay for Piv DNA binding sites within the inversion region was carried out using gel electrophoresis mobility shift assays (EMSA; data not shown) with a series of restriction fragments that spanned both the invertible segment and the adjacent sequences required for inversion in E. coli (Fig. 3A). Several of the fragments were shifted in the presence of MBP-Piv (+ in Fig. 3A). Two fragments, 430 and 870 bp, were chosen for further analysis by DNase I footprinting. The 430-bp fragment was selected because it spans the left recombination site of the invertible element (invL). The 870-bp fragment also contains the invL recombination site, plus a potential accessory site (see below).

DNase I protection assays with MBP-Piv and the 870-bp fragment showed protections and enhancements from about −285 to −265 bp upstream of the invL cross-over site (data not shown). However, due to the relatively large size of the fragment, the DNase I cleavage pattern at the site was not easily resolved. Therefore, a smaller fragment of 298 bp, containing the protected DNA sequence, was examined by DNase I protection assays (Fig. 3, B and C).

The DNase I cleavage pattern in the MBP-Piv binding region has long tracts of no cleavage due to A-tract repeats that narrow the minor groove, thus inhibiting DNase I binding and cleavage (15). These A-tracks are also appropriately phased along the DNA helix to contribute sequence-directed DNA bending to this region of the DNA (16). Protections and enhancements from −233 to −247 and from −266 to −285 (Fig. 3, B and C) could be resolved in these DNase I protection assays. No other protected regions were detected on the 298-bp fragment. This MBP-Piv binding site is probably too distant from the site of DNA cleavage (+1) and strand exchange in the inversion reaction to be the binding site for the Piv subunit that actually catalyzes the recombination reaction. However, it is likely that this Piv binding site is an accessory site utilized in
the assembly of an active synaptic complex for Piv-mediated DNA inversion (see “Discussion”). The 430-bp fragment (Fig. 3A) contains the 26-bp site of inversion (invL) that was defined based upon sequence homology at the ends of the invertible segment (17). The initial EMSA analysis of MBP-Piv binding to the 430-bp fragment indicated that MBP-Piv does bind specifically, albeit weakly, to a sequence within this fragment (data not shown). However, DNase I and 1,10-phenanthroline-copper protection assays with the 430-bp fragment and MBP-Piv showed no evidence of protein-DNA interactions (data not shown). Because MBP-Piv does shift the 430-bp fragment in EMSA gels, indirect DNase I protection assays were performed with the 430-bp fragment in which the electrophoretically separated protein-DNA complex and unbound DNA are treated with DNase I in the non-denaturing polyacrylamide gel and then isolated from gel slices for electrophoresis on the DNA sequencing polyacrylamide gel (see “Materials and Methods”). Even though this assay should have enriched for DNA complexes with MBP-Piv, no protection of DNA sequence by MBP-Piv was observed. These results suggested that the binding of MBP-Piv to the 430-bp fragment was either unstable or not specific.

To determine if the poor interactions of MBP-Piv with invL are due to the MBP protein at the amino terminus of Piv, Piv was tagged at the COOH terminus with six histidines (see “Materials and Methods”). The Piv-His6 fusion protein was partially purified and used in EMSA and DNase I protection assays with the 430-bp fragment and also with the 298-bp fragment containing the accessory Piv binding site (data not shown). All of the DNA binding results with Piv-His6 corresponded exactly with those obtained with MBP-Piv, indicating that the poor binding of MBP-Piv to invL is not due to steric hindrance by MBP of Piv-specific binding. Therefore, stable binding of Piv may require other factors such as accessory DNA binding sites, accessory proteins, and/or supercoiled DNA substrate to facilitate binding to the recombination sites (see “Discussion”).

**Piv Binds Two Different Recognition Sequences**—To characterize the interactions of MBP-Piv with the isolated invL and upstream accessory site, double-stranded oligonucleotides corresponding to each site were synthesized (designated INV and SUB, respectively; Fig. 4A). The sequence of INV included the 26-bp region of homology from either end of the invertible element and 14 bp of sequence immediately upstream and downstream from the invL site. The DNA sequence of SUB (or strong upstream binding site oligonucleotide) was designed based on the DNase I footprint originally obtained with the 870-bp DNA fragment (i.e., the protected region −269 to −284, but not the −233 to −245 region). The INV and SUB DNA binding substrates were used in an EMSA to examine MBP-Piv binding at each site (Fig. 4B).

Two shifted complexes (S-1 and S-2, lane 2, Fig. 4B) were formed with the SUB oligonucleotide, indicating that at least two MBP-Piv protomers bind SUB. Because the SUB oligonucleotide does not include the DNA sequence from −233 to −247 bp (see Fig. 3), we have designated the MBP-Piv binding site encoded within the SUB oligonucleotide, sub1.

MBP-Piv binding to INV resulted in one primary complex (I-1) that migrated at approximately the same rate as the slower migrating complex formed with SUB (S-2). Because the difference in the molecular weights of the INV and SUB oligonucleotides is nominal in comparison to the MBP-Piv molecular weight, this result suggests that the I-1 and S-2 protein-DNA complexes contain the same number of MBP-Piv protomers. No faster migrating protein-DNA complex was seen with INV when the concentration of MBP-Piv was decreased (data not shown). The presence of a slower complex (I-2) was detected with some variability in the EMSAs. The formation of this complex suggests that a higher multimeric MBP-Piv may bind INV or an additional INV oligonucleotide may bind the I-1 complex.

Based upon the predicted molecular weights of INV, SUB, and MBP-Piv, the electrophoretic mobilities of I-1, S-1, and S-2 indicate that INV is bound by at least two MBP-Piv protomers while SUB is bound by either single (S-1) or multiple (S-2) MBP-Piv protomers. Comparison of the Piv DNA binding substrates, INV and SUB, revealed very little homology (TATNC), suggesting that Piv has two different DNA recognition sites.

**Specificity of MBP-Piv Binding to INV and SUB**—To further examine the interactions of MBP-Piv with the apparently different binding sites on INV and SUB, competition assays were performed using each of the oligonucleotides as both binding substrates and competitor DNA. In Fig. 5A, the SUB oligonucleotide was labeled and used in DNA binding assays with MBP-Piv and the unlabeled competitor DNAs: SUB, INV, and the nonspecific oligonucleotide (see “Materials and Methods.”). Although SUB competed for both S-1 and S-2 complex formation at 100- and 500-fold excess unlabeled oligonucleotide, INV...
The binding of MBP-Piv to the site of inversion was cooperative as indicated by the slope of the line in the graph. The dissociation rate of I-1 and S-2 is also consistent with multimerization of MBP-Piv producing an unstable protein complex.

Examination of the Cooperativity of Piv Binding—Cooperative interactions stimulate binding of multiple proteins to DNA sites. Therefore, cooperative binding of MBP-Piv may facilitate complex formation with the inv or sub sites where earlier results suggest that multiple protomers of MBP-Piv are binding. However, a multimeric form of MBP-Piv may bind noncooperatively if assembled in solution before binding to the inv or sub sites. To determine cooperativity, ln[P] versus ln ([PX]/[X]) was plotted producing a slope n, where [P] is the concentration of MBP-Piv protein added, and [PX] and [X] are the concentrations of the bound and unbound DNA, respectively. For example, if MBP-Piv binds as a dimer to the DNA, n equals 2 indicates that the binding of the two proteins is cooperative; n equals 1 indicates that the binding is not cooperative, and therefore, the dimer is forming before binding to that DNA site.

To determine the cooperativity of MBP-Piv binding in the S-1 and S-2 complexes, increasing amounts of MBP-Piv were incubated with the 298-bp DNA fragment encoding the full sub site and subsequently analyzed by EMSA. Focusing on the MBP-Piv concentrations that gave the S-1 complex, the ratio of bound to unbound DNA was determined and compared with the concentration of MBP-Piv used. The ln[P] versus the ln([PX]/[X]) was plotted for binding reactions with 0.63 to 31.6 nM MBP-Piv (Fig. 7A). The slope of the resulting graph is 1, showing noncooperative binding by MBP-Piv at sub in the S-1 complex. Analysis of the S-2 complex revealed that Piv is binding cooperatively. The ln[P] versus the ln([PX]/[X]) was plotted for binding reactions with 47.4 to 1580 nM MBP-Piv (Fig. 7A), and the resulting graph has a slope of 2, indicating that the binding is cooperative. This result supports the conclusion that at least two MBP-Piv proteins are binding to the sub site in the S-2 complex.

Cooperativity was also examined for Piv binding to the site of inversion using the INV oligonucleotide (Fig. 7B). The I-1 complex competed effectively for only S-2 complex formation at the same molar excess of competitor DNA. This result is consistent with MBP-Piv interacting with invL only in a multimeric form (Fig. 4). The nonspecific DNA competitor also competed (less effectively) with MBP-Piv binding to give S-2 complex. This competition for S-2 complex formation by the nonspecific competitor may indicate that multimeric MBP-Piv protein has a faster off rate and so it is titrated out of the reaction by nonspecific protein-DNA interactions at 100–500-fold excess nonspecific competitor DNA. Fig. 5A shows that only the INV oligonucleotide effectively competed for MBP-Piv binding to labeled INV; SUB did not show significant competition even at 500-fold molar excess. These results again suggest a different interaction between MBP-Piv and invL as compared with binding of MBP-Piv to invL.

Dissociation Rate Analysis—The results of the DNase I footprinting, gel shift assays, and DNA binding competition analyses could be interpreted as indicating that the interactions of MBP-Piv with inv are less stable than with sub. The inability to obtain more than 40% of the INV DNA bound with increasing concentrations of MBP-Piv might be explained by a rapid dissociation rate for MBP-Piv when bound to the site of inversion, such that no more than 40% of the INV oligonucleotide complexed with MBP-Piv can be detected by the DNA binding assays used. Dissociation rate experiments with INV (Fig. 6A), using 5000-fold molar excess of competitor (unlabeled INV), showed that the percent bound DNA in the I-1 complex decreased >50% after 15 s of incubation with the competitor oligonucleotide. Surprisingly, even though 5000-fold excess INV competitor DNA prevented any complex formation when added before MBP-Piv (control lane in Fig. 6A), there was still close to 10% I-1 complex after a 1-h incubation of the preformed MBP-Piv-INV complex with 5000-fold excess unlabeled INV. The dissociation rate experiments with SUB (Fig. 6B) gave a half-life for the S-2 complex of about 15 s, similar to the I-1 complex. The S-1 complex, however, had a much higher half-life of 12 min, indicating that MBP-Piv binding as a single protomer in this complex is more stable. The apparent rapid dissociation rate of I-1 and S-2 is also consistent with multimerization of MBP-Piv producing an unstable protein complex.

FIG. 5. Specificity of MBP-Piv binding to the INV and SUB oligonucleotides. Competition DNA binding assays were performed as described under “Materials and Methods” for both labeled INV and SUB. A, an EMSA gel is shown on which reactions containing 32P-labeled SUB oligonucleotide, 32 nM MBP-Piv protein, and cold competitor DNA were electrophoresed. The fold molar excess of competitor DNA is indicated above each lane, and the lanes are grouped according to the competitor DNA used: INV, SUB, or nonspecific cold competitor DNA. Probe indicates the lane where no protein was added to the reaction. B, competition assays are shown in which each DNA binding reaction contained 32P-labeled INV oligonucleotide (see “Materials and Methods”), 630 nmol of MBP-Piv protein, and cold competitor DNA as indicated. The lanes are labeled as described in A, S-1, S-2, and I-1 indicate the same complexes as in Fig. 4, and U is the unbound DNA binding substrate. The nonspecific competitor oligonucleotide has no homology to the INV or SUB oligonucleotides.
plex accumulated with increasing amounts of Piv. Again, no intermediate protein-DNA complex was seen. The ln[P] versus ln([PX]/[X]) was plotted for each lane, and the slope of the resulting graph is 0.6; this result indicates that noncooperative binding is occurring. Therefore, multimeric MBP-Piv must assemble in solution or at another site before binding to invL.

**FIG. 6.** Dissociation rates of MBP-Piv complexed with INV and SUB. Gel shift DNA binding assays are shown for protein-DNA complexes that were challenged with 5000-fold molar excess of competitor DNA. The percentage of bound DNA in each complex at each time point is represented on a graph. A, a dissociation rate assay performed with labeled INV produces a graph showing that the MBP-Piv protein bound in the I-1 complex has a half-life of about 15 s. B, a dissociation rate assay with SUB gives a result for both the S-1 (12-min half-life) and S-2 (15-s half-life) complexes as determined from the graph below the gel. S1, S2, and I-1 indicate the same complexes as in Fig. 4.

**FIG. 7.** Cooperativity of MBP-Piv binding to the invL and sub sites. A, an EMSA gel is shown in which the labeled 298-bp fragment is incubated with increasing amounts of MBP-Piv. The results for the S-1 and S-2 complexes (see Fig. 4) are plotted as ln[P] versus ln([PX]/[X]), as described under “Results,” for the range of MBP-Piv concentration that gives a single retarded complex and the range that gives two retarded complexes, respectively. B, the binding of MBP-Piv to the INV oligonucleotide is examined, and the results for the I-1 complex are plotted as above.

**DISCUSSION**

The site-specific recombinase Piv is a member of the novel family of DNA recombinases that includes the IS110/IS492 transposases (5). To determine the mechanism for DNA cleavage and strand exchange mediated by this unique DNA inverter, we must understand how Piv interacts with the chromosomal DNA segment that it inverts in M. lacunata. We have presented here DNA binding studies with MBP-Piv and DNA sequences from the type IV pilin inversion region of M. lacunata. The activity of the MBP-Piv fusion protein in these gel
electrophoresis DNA binding assays and nuclease/chemical protection analyses is likely to correlate to the activity of wild type Piv, as MBP-Piv is active \( \textit{in vivo} \) for DNA inversion (see Fig. 2).

Previous examination of the DNA sequence of the inversion region of \( \textit{M. lacunata} \) revealed 26-bp inverted repeats, \( \textit{invL} \) and \( \textit{invR} \), which defined the left and right ends of the invertible element (17, 18). Piv-mediated DNA cleavage and strand exchange must occur within these two 26-bp sequences for site-specific DNA inversion of the pilin segment. Therefore, based on other site-specific recombination and transposition systems (1, 2), it seemed likely that Piv binds to a DNA sequence overlapping or adjacent to \( \textit{invL} \) and \( \textit{invR} \). The results of electrophoretic mobility shift assays indicate that MBP-Piv does indeed interact with DNA fragments containing both \( \textit{invL} \) and \( \textit{invR} \), as well as with a few DNA fragments from both outside and within the invertible DNA segment of \( \textit{M. lacunata} \) (see Fig. 3A). The binding of MBP-Piv to \( \textit{invL} \) and one of the additional sites located outside of the invertible segment was further characterized.

MBP-Piv interactions with the \( \textit{invL} \) site are quite weak, as indicated by the gel shift DNA binding assays in which less than 40% of the labeled DNA substrate (54-bp double-stranded INV oligonucleotide or 430-bp DNA fragment) could be specifically bound, no matter how high the concentration of MBP-Piv protein (see Fig. 7B and data not shown). In addition, MBP-Piv did not give a “footprint” at the \( \textit{invL} \) site in either nuclease or chemical protection assays. Measurements of the dissociation rate for the MBP-Piv bound to \( \textit{invL} \) (on INV, see Fig. 6A; or the 430-bp fragment, data not shown) show that the complex is unstable; the half-life of the complex is less than 15 s. While this weak binding of MBP-Piv to \( \textit{invL} \) could have been due to the presence of the maltose-binding protein at the amino terminus of Piv, we found that Piv containing a small His\(_6\) tag at the COOH terminus also bound \( \textit{invL} \) very weakly.

Piv is certainly not the first site-specific recombinase to exhibit poor affinity for the site where it mediates DNA cleavage and strand exchange. \( \lambda \) integrase (Int) binds very poorly to the “core” sites where it mediates DNA cleavage and strand exchange at the \( \textit{attP/attB} \) or \( \textit{attL/attR} \) sites. Cooperative interactions with Int bound to the “arm-type” sites and DNA bending mediated by IHF facilitate binding of Int to the core sites of \( \textit{attP} \) and \( \textit{attL} \) (19, 20). Int binds with very low affinity to the \( \textit{attB} \) core sites (21); indeed, Int does not give a DNase I footprint at the core site of \( \textit{attR} \), even in the presence of the accessory proteins and arm-type sites (19).

\( \lambda \) Int is a bivalent protein, having two different, separable DNA binding domains. The Int amino-terminal domain binds with high affinity to the arm-type sites, while the carboxy-terminal domain binds weakly to the core sites and catalyzes DNA cleavage and strand exchange. Thus, monomers of Int can bridge the arm and core sites within \( \textit{att} \) sites and bring \( \textit{att} \) sites together in synapsis (20, 22). Because Piv binds so poorly to the \( \textit{invL} \) site, we did not find it surprising that, like Int, Piv binds other DNA sequences that may facilitate the formation of a synaptic complex. The nuclease protection and gel shift DNA binding assays show that Piv binds with higher affinity to a different DNA recognition sequence located upstream of \( \textit{invL} \) (Figs. 3, B and C, 4A, and 7, A and B). This strong upstream binding site (\( \textit{sub} \)) is bound cooperatively by at least two protomers of MBP-Piv (see Figs. 4B and 7A). The dissociation rate assays (see Fig. 6B) performed with the \( \textit{sub} \) site suggest that the first MBP-Piv protomer that binds to \( \textit{sub} \) forms a stable complex (S-1) but the multimeric complex (S-2) is less stable.

The interactions of MBP-Piv with \( \textit{invL} \) appear to differ from those with \( \textit{sub} \), not only in the affinity and stability, but also in the form of the protein required for binding to the site. The cooperativity assays and competition analyses suggest that only the multimeric form of MBP-Piv binds to \( \textit{invL} \), while single protomers of MBP-Piv bind cooperatively to \( \textit{sub} \) (see Figs. 5 and 7). Again, this is not unusual behavior for a bivalent protein that mediates DNA recombination. The transposase of bacteriophage Mu, MuA, binds as a monomer to sites within the transpositional enhancer sequence which facilitates assembly of a synaptic structure (23, 24). But, MuA recognizes the recombination sites at the ends of the transposable element with a different domain of the protein, and then assembles a tetramer that is bound to both ends of the element on a supercoiled DNA substrate (25, 26). The interactions of MuA with the end recombination sites actually promote tetramer formation, and it is this complex that is active for recombination (27).

In fact, under altered reaction conditions MuA can form a tetramer upon binding a single recombination site, but not on the enhancer sites (27). Therefore, when we consider the nature of the MBP-Piv complexes with \( \textit{sub} \) and \( \textit{invL} \), it is possible that the \( \textit{sub} \) site is an assembly site for the multimeric form of Piv that binds to the recombination sites. The instability of the MBP-Piv multimeric complex with \( \textit{invL} \) may reflect the need for stabilization within a correct synaptic complex, like the binding of \( \lambda \) integrase to the core sites, or, perhaps Piv transiently interacts with the \( \textit{inv} \) sites within an invertasome-like structure and the multimeric complex rapidly falls apart following catalysis of the recombination reaction. As has been suggested for a number of transposition and site-specific recombination systems (Ref. 28; for reviews see Refs. 1 and 2), it is undesirable for the recombinase to be active at recombination sites until a synaptic structure is assembled; this ensures that the correct strand exchange and religation occurs after DNA cleavage.

If \( \textit{sub} \) acts as an assembly site for Piv, it is likely to stimulate the rate of inversion or to be required for Piv-mediated DNA inversion. Previous deletion analyses of the \( \textit{M. lacunata} \) invertible segment and surrounding DNA sequences have shown that the region encoding the \( \textit{sub} \) site is not essential for inversion of the \( \textit{Moraxella} \) DNA segment in \( \textit{E. coli} \), although it may have an affect on the rate of inversion (14). Like the P2 arm site in \( \lambda \textit{attR} \) (22), the \( \textit{sub} \) site may be stimulatory only under certain conditions, such as when recombinase levels are low or in the presence/absence of accessory factors. Analysis of the nucleotide sequence of the inversion region using the GCG Wisconsin Package Bestfit program with the \( \textit{sub} \) site sequence revealed two other possible sites within the 535-bp and 250-bp DNA fragments that were bound by MBP-Piv in the gel shift DNA binding assays (see Fig. 3A; data not shown). These other sites may substitute for the \( \textit{sub} \) site or coordinate to regulate the inversion reaction with \( \textit{sub} \). In order to define the roles of Piv and its different binding sites in the inversion reaction, we are currently using \( \textit{in vitro} \) recombination assays to isolate intermediates in the DNA inversion reaction.

Acknowledgments—We thank Gordon Churchward, Russell Karls, Charles Moran, and Maureen Powers for critical review of this manuscript and helpful discussions. We are grateful to Jeff Mahr for his contribution to the construction of pAG862.

REFERENCES
1. Craig, N. L. (1996) in \textit{Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology} (Neidhardt, F. C., ed) 2nd Ed., Vol. 2, pp. 2339–2362, American Society for Microbiology, Washington, DC.
2. Hallet, B., and Sherratt, D. J. (1997) \textit{FEBS Microbiol. Rev.} 21, 157–178.
3. Landy, A. (1993) \textit{Curr. Opin. Genet. Dev.} 3, 699–707.
4. Mahillon, J., and Chandler, M. (1998) \textit{Microbiol. Mol. Biol. Rev.} 62, 725–774.
5. Lenich, A. G., and Glasgow, A. C. (1994) \textit{J. Bacteriol.} 176, 4160–4164.
6. Marx, C. F., Roza, P. W., Hackel, M., Stevens, S. F., and Glasgow, A. C. (1990) \textit{J. Bacteriol.} 172, 4370–4377.
7. di Guan, C., Li, P., Riggs, P. D., and Inouye, H. (1988) \textit{Gene} \textit{(Amst.)} 67, 21–30.
8. Maina, C. V., Riggs, P. D., Grandea, A. G. d., Slature, B. E., Moran, L. S.,...
DNA Binding Activities of the Novel Recombinase, Piv

Tagliamonte, J. A., McReynolds, L. A., and Guan, C. D. (1988) *Gene (Amst.)* 74, 365–373
9. Sambrook, J., Fritsch, E. F., and Maniatis, T. (eds) (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
10. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1991) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York
11. Glasgow, A. C., Bruist, M. F., and Simon, M. I. (1989) *J. Biol. Chem.* 264, 10072–10082
12. Maxam, A. M., and Gilbert, W. (1980) *Methods Enzymol.* 65, 499–560
13. Kuwabara, M. D., and Sigman, D. S. (1987) *Biochemistry* 26, 7234–7238
14. Kristensen, H. H., Valentin-Hansen, P., and Sogaard-Andersen, L. (1996) *J. Mol. Biol.* 260, 113–119
15. Drew, H. R., and Travers, A. A. (1984) *Cell* 37, 491–502
16. Koo, H. S., Drak, J., Rice, J. A., and Crothers, D. M. (1990) *Biochemistry* 29, 4227–4234
17. Fulks, K. A., Marrs, C. F., Stevens, S. P., and Green, M. R. (1990) *J. Bacteriol.* 172, 310–316
18. Rozsa, F., and Marrs, C. (1991) *J. Bacteriol.* 173, 4090–4096
19. Thompson, J. F., Moitoso de Vargas, L., Skinner, S. E., and Landy, A. (1987) *J. Mol. Biol.* 195, 481–493
20. Kim, S., Moitoso de Vargas, L., Nunes-Duby, S. E., and Landy, A. (1990) *Cell* 63, 773–781
21. Ross, W., and Landy, A. (1983) *Cell* 33, 261–272
22. Richet, E., Abcarian, P., and Nash, H. A. (1988) *Cell* 52, 9–17
23. Surette, M. G., and Chaconas, G. (1992) *Cell* 68, 1101–1108
24. Mizuuchi, M., Baker, T., and Mizuuchi, K. (1992) *Cell* 70, 363–311
25. Surette, M. G., Buch, S. J., and Chaconas, G. (1987) *Cell* 49, 253–262
26. Craigie, R., and Mizuuchi, K. (1987) *Cell* 51, 493–501
27. Baker, T., and Mizuuchi, K. (1992) *Genes Dev.* 6, 2221–2232
28. Merickel, S. K., Haykinson, M. J., and Johnson, R. C. (1998) *Genes Dev.* 12, 2863–2876