

Phage Bxb1 integrase mediates highly efficient site-specific recombination in mammalian cells

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We are interested in identifying and characterizing recombinases that can achieve efficient site-specific insertion or cassette exchange in mammalian cells. The combination of gene targeting techniques and site-specific recombination systems (Cre/loxP and Flp/FRT) have been widely exploited in genetic analysis and in engineering complex chromosomal alterations in higher eukaryotes (1). The Cre/loxP system has been the preferred tool for genome engineering in murine embryonic stem cells (ESCs) and in mice. Cre and Flp are best suited for generating site-specific deletions (knockouts), because the 34-bp loxP and FRT recombination target sites, respectively, are recreated during recombination, and excision is preferred over integration. Mutant loxP sites have been developed to stabilize the integration event, but the efficiency of integration is still low (2). Attempts to exploit integrases from λ phage and closely related HK022 phage have resulted in limited success because of cofactor requirement and poor efficiency of intermolecular integration and intrachromosomal deletion (3,4). Recent work on integrases from Φ C31, R4, and TP901-1 phages demonstrated that these enzymes catalyze site-specific recombination between *attP* (phage attachment) and *attB* (bacterial attachment) sites in mammalian cells (5–8). These integrases belong to the resolvase/invertase or serine recombinase family that utilizes an N-terminal catalytic serine to mediate recombination and are structurally different from Cre, Flp, and λ *int* of the λ integrase or tyrosine recombinase family that utilizes a C-terminal catalytic tyrosine (9).

Recently it was shown that phage Bxb1 integrates into *Mycobacterium smegmatis* *groEL1* gene, and Bxb1

integrase can catalyze recombination between the *attP* and *attB* sites in vitro in the absence of supercoiled DNA, cofactors, and divalent cations (10–13). Here we report that Bxb1 integrase, a serine recombinase, is functional in mammalian cells and catalyzes highly efficient unidirectional recombination between short heterologous *attP* and *attB* target sites resulting in the integration or deletion of DNA depending on the orientation and location of *attP* and *attB* sites.

To determine if the Bxb1 integrase functions in mammalian cells, we created a recombination assay plasmid pCMV-*attP/attB* containing a transcription termination or stop sequence, flanked by 52-bp *attP* and 46-bp *attB* sites (10), placed between the cytomegalovirus (CMV) promoter and the luciferase reporter gene (Figure 1A). Recombination between the *attP* and *attB* sites catalyzed by Bxb1 integrase would result in deletion of the stop sequence and activation of luciferase gene. Since the integrase gene is from bacteriophage, we totally synthesized the gene using codons optimized for high-level human and mouse expression without changing the encoded amino acid sequence (GenBank® accession no. NP_075302) and cloned it into a CMV promoter-driven mammalian expression plasmid to obtain pCMV-Bxb1. To test the recombination in various cell types, we obtained and cultured HEK 293 (ATCC, Manassas, VA, USA), NIH 3T3 (ATCC), mouse 129/S6 ESCs (Primogenix, St. Louis, MO, USA), mouse C17.2 neural stem cells (NSCs; Evan Snyder, The Burnham Institute, La Jolla, CA, USA), and rat bone marrow stromal cells (BMSCs; Osiris Therapeutics, Baltimore, MD, USA) following the recommended protocols.

One day before transfection, cells were plated in a 96-well (HEK 293, NIH 3T3) or 48-well plate (BMSC, ESC, NSC) at different densities depending on the cell type (HEK 293, 20,000 cells; NIH 3T3, 5000 cells; ESC, 10,000 cells, NSC, 120,000 cells; and BMSC, 3200 cells). The cells were then transfected with 25 ng recombination assay plasmid alone or along with varying amounts of pCMV-Bxb1 DNA (0, 25, 50, or 100 ng) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Constitutively expressed *Renilla* luciferase reporter plasmid (pRL-CMV; Promega, Madison, WI, USA) was cotransfected (2 ng/well) and used as an internal control to normalize the transfection efficiency. Twenty-four hours (HEK 293, NIH 3T3, ESC) or 48 h (BMSC, NSC) after transfection, the media was discarded, and the cells were lysed with 50 μ L passive lysis buffer (Promega) and 25- μ L extracts were then assayed using the Dual Luciferase® Assay kit (Promega) on a plate reader equipped with injectors (Dynex Technologies, Chantilly, VA, USA). Introduction of pCMV-*attP/attB* and pCMV-Bxb1 plasmid DNAs into human HEK 293 cells, mouse NIH 3T3, NSCs, and ESCs, and rat BMSCs resulted in 215- to 2886-fold induction of luciferase activity depending on the cell type (Figure 1B, only the values obtained for 100 ng pCMV-Bxb1 are shown). Thus, the results clearly show that the Bxb1 integrase can catalyze the site-specific recombination reaction in mammalian cells, and its activity in cells from three different species suggests that the integrase may function autonomously of cellular factors.

The ability of the Bxb1 integrase to catalyze recombination between chromosomally placed *attB* and *attP* sites was assessed by stably integrating a single copy of CMVp-*attP*-STOP-*attB*-luciferase sequence (Figure 1A) at the FRT locus in Flp-In™-293 cells (Invitrogen) following the manufacturer's instructions and transiently introducing pCMV-Bxb1. To place a single copy of CMVp-*attP*-STOP-*attB*-luciferase sequence in Flp-In-293 cells, we first cloned the sequence into the pcDNA5/FRT plasmid (Invitrogen) to create pFRT-*attP/attB* and then

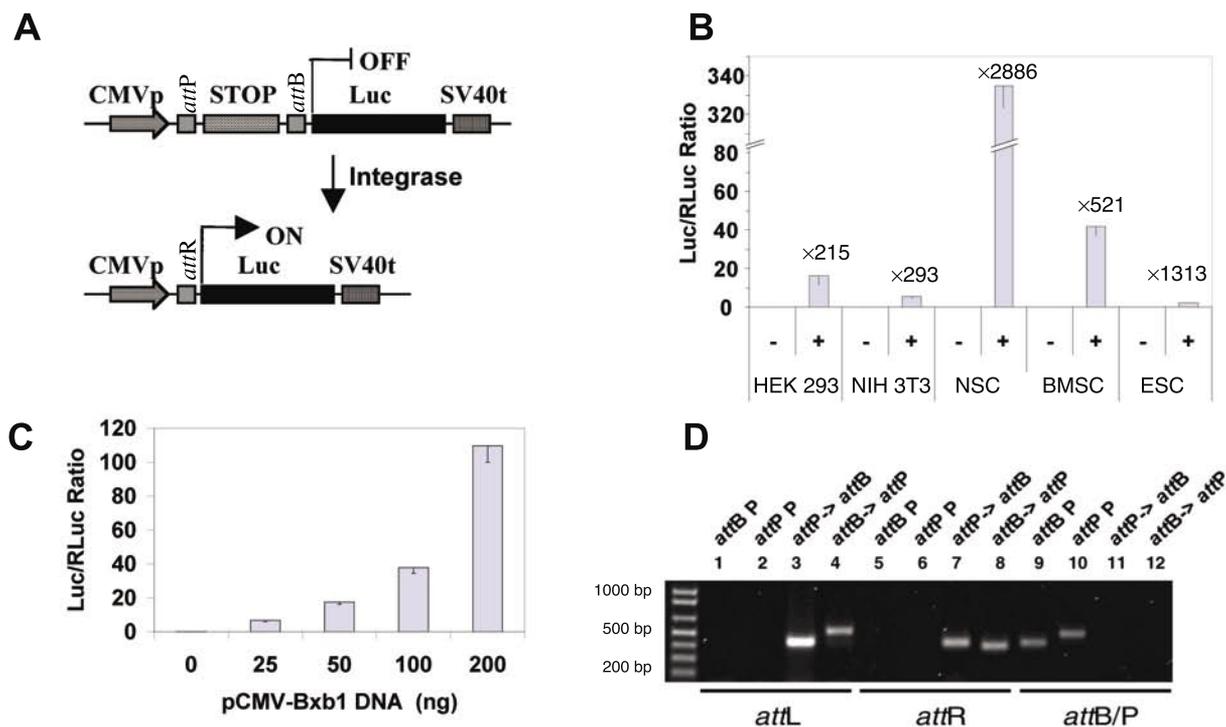


Figure 1. Site-specific recombination activity of Bxb1 phage integrase in mammalian cells. (A) Representation of pCMV-attP/attB construct and activation of luciferase gene expression after deletion of stop sequence. (B) Bxb1 integrase activity in human HEK 293 ($n = 6$), mouse NIH 3T3 ($n = 5$), mouse embryonic stem cells (ESCs; $n = 3$), mouse neural stem cells (NSCs; $n = 4$), and rat bone marrow stromal cells (BMSCs; $n = 8$). Cells were cotransfected with 2 ng *Renilla* luciferase (RLuc) plasmid pRL-CMV and 25 ng pCMV-attP/attB plasmid in the absence (-) or presence (+) of 100 ng pCMV-Bxb1 plasmid. Luciferase (Luc) and RLuc activities were assayed after 48 h incubation using the Dual Luciferase Assay kit reagents and a luminometer equipped with injectors. Values shown are the mean \pm SD of ratios of Luc and RLuc activities. Fold-inductions (ratio of activity in the presence of pCMV-Bxb1 to the activity in the absence of pCMV-Bxb1) are shown above the bars. (C) Deletion of chromosomal DNA flanked by attP and attB sites. Luciferase activity in Flp-In-293 cells stably transfected with pCMV-attP/attB and transiently cotransfected with the indicated amounts of pCMV-Bxb1 plasmid along with 2 ng pRL-CMV plasmid. Values were measured 48 h posttransfection and are the mean \pm SD ($n = 8$) of ratios of Luc and RLuc activities. (D) Integration-specific PCR analysis. Confirmation of attL and attR sites in clones after the integration of pPURO-attB into attP cells (attB > attP, lanes 4, 8, and 12) and integration of pPURO-attP into attB cells (attP > attB, lanes 3, 7, and 11), and attB and attP sites present in the cells before the integration (attB, lanes 1, 5, and 9; attP, lanes 2, 6, and 10) is achieved by primer combinations that amplify attL (lanes 1–4), attR (lanes 5–8), and attB and attP (lanes 9–12) sites. CMVp, SV40t, and Luc are cytomegalovirus (CMV) promoter, simian virus 40 (SV40) terminator, and luciferase gene, respectively.

cotransfected Flp-In-293 cells with pFRT-attP/attB DNA and Flp recombinase expression plasmid pOG44 DNA. We then selected several stable clones that were resistant to hygromycin and sensitive to zeocin and that lost β -galactosidase. A representation of the FRT locus is shown in Supplementary Figure S1B available online at www.BioTechniques.com. Cells derived from a single stable clone (15,000 cells/well, 96-well plate) were then transfected with 0, 25, 50, 100, or 200 ng pCMV-Bxb1, and the luciferase activity was measured after 48 h of incubation as described above. As shown in Figure 1C, increasing amounts of the pCMV-Bxb1 DNA resulted in a dose-dependent increase in luciferase expression. The Bxb1 integrase, therefore, functioned within the environment of the mammalian

cell nucleus and deleted chromosomal DNA flanked by attP and attB sites.

In order to test the ability of the integrase to insert a circular plasmid DNA containing an attP site into an attB site present on the chromosome (or an attB site into an attP site), we made Flp-In-293 stable cell lines containing a single copy of the CMV promoter and an attB site (or the CMV promoter and an attP site) at the FRT locus. First, we inserted the 46-bp attB or 52-bp attP oligonucleotide after the CMV promoter in pcDNA5/FRT to make plasmids pFRT-attB or pFRT-attP. Next, a single copy of either pFRT-attB or pFRT-attP was introduced at the FRT locus of Flp-In-293 cells as described above to create two sets of stable clones. Two integration assay plasmids, pPURO-attP and pPURO-attB, were then constructed in which

an attP or attB site was placed before a promoter-less puromycin resistance gene. Site-specific recombination between pPURO-attP and the chromosomal attB site (or pPURO-attB and the chromosomal attP site) would place the promoter-less puromycin gene next to the CMV promoter and confer a drug resistance phenotype to the cells. Random integration of pPURO-attP or pPURO-attB is not expected to yield any drug-resistant clones, unless the integration is next to a promoter. A representation of the FRT locus before and after the integration of puromycin resistance gene is shown in Supplementary Figure S1C.

Cells derived from a single stable clone containing either pFRT-attB or pFRT-attP (500,000 cells/well, 6-well plate) were then transfected with either 1 μ g pPURO-attP or pPURO-attB alone

or along with 4 μg pCMV-Bxb1. CMV promoter-driven green fluorescent protein (GFP) plasmid DNA (1 μg) was transfected, and the GFP positive cells were counted 48 h after transfection to estimate the transfection efficiency. The transfection efficiency varied from 40%–70% in these experiments. Stable cells were then selected on medium containing either puromycin (3 $\mu\text{g}/\text{mL}$, for selecting stable integrations at *attP* or *attB* sites) or hygromycin (150 $\mu\text{g}/\text{mL}$, for selecting stable integrations at FRT site). Cotransfection of pPURO-*attP* and pCMV-Bxb1 into Flp-In-293 stable cells containing *attB* site resulted in 67% ($n = 4$, $\text{SD} = 10$) of cells that took up the DNA becoming stable drug-resistant clones. Similarly, 98% ($n = 4$, $\text{SD} = 9$) of transfected cells developed into stable clones after cotransfection of Flp-In-293 cells containing an *attP* site with pPURO-*attB* and pCMV-Bxb1. Introduction of pPURO-*attP* + pCMV-Bxb1 plasmids into cells containing an *attP* site or pPURO-*attB* + pCMV-Bxb1 plasmids into cells containing an *attB* site produced only two drug-resistant clones, showing that integrase only recombines *attP* and *attB* sites. As a comparison, under identical experimental conditions, we observed 15% ($n = 4$, $\text{SD} = 2$) of transfected cells becoming stable drug-resistant clones after targeting the pcDNA5/FRT plasmid into Flp-In-293 cells with an FRT site in the presence of Flp recombinase expression plasmid pOG44. Recombination between *attP* and *attB* sites creates *attL* and *attR* sites, which are hybrid sites between *attP* and *attB* sites (12). To confirm site-specific integration, PCR analysis was done to detect *attL* and *attR* sites using specific primers designed to amplify the sites. As predicted, PCR analysis showed the presence of *attL* and *attR* sites and the absence of *attP* or *attB* sites in the genomic DNA isolated from pooled drug-resistant clones (Figure 1D). Recovered *attL* and *attR* PCR fragments had exact nucleotide sequences of the expected *attL* and *attR* sites (data not shown). These results clearly indicate that Bxb1 integrase is highly efficient and superior to Flp recombinase in integrating plasmid DNA at chromosomal *attP* and *attB* sites.

In conclusion, we show that Bxb1 integrase is functional in mouse, human, and rat cells, can efficiently integrate circular DNA into a chromosome, and can delete chromosomal DNA, depending on the location of the *att* sites. Since integrases have no theoretical limit on the size of the DNA they can insert, bacterial artificial chromosomes (BACs) and other large clones can be integrated into the genome. In previous work, ΦC31 integrase was used to integrate an *attP*-containing plasmid into pseudo-attachment sites present on human and mouse chromosomes (14). Our preliminary experiments suggest that Bxb1 integrase does not integrate plasmids with *attP* or *attB* into pseudo-attachment sites in human HEK 293 and mouse NIH 3T3 cells, and it can be stably expressed in mouse ESCs after targeting to the *hprt* locus. Bxb1 integrase may be an ideal choice for integration or cassette exchange in ESCs and for applications in animals where efficient unidirectional recombination is required because of highly efficient integration at *att* sites and the lack of integration at pseudo *att* sites.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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