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Prototype foamy virus integrase is promiscuous for target choice

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Abstract

Retroviruses have two essential activities: reverse transcription and integration. The viral protein integrase (IN) covalently joins the viral cDNA genome to the host DNA. Prototype foamy virus (PFV) IN has become a model of retroviral intasome structure. However, this retroviral IN has not been well-characterized biochemically. Here we compare PFV IN to previously reported HIV-1 IN activities and discover significant differences. PFV IN is able to utilize the divalent cation calcium during strand transfer while HIV-1 IN is not. HIV-1 IN was shown to completely commit to a target DNA within 1 min, while PFV IN is not fully committed after 60 min. These results suggest that PFV IN is more promiscuous compared to HIV-1 IN in terms of divalent cation and target commitment.

Keywords

Prototype foamy virus; Integrase; Enzymology; Divalent cation; Retrovirus

1. Introduction

Retroviral integration is the covalent insertion of the viral cDNA genome into the host DNA [1]. This essential step of the retroviral life cycle is performed by the viral enzyme integrase (IN). Following entry to a cell, reverse transcriptase copies the viral genomic RNA to a linear double stranded cDNA. IN must assemble onto the two ends of the viral cDNA before it completes two enzymatic activities. First, IN cleaves two nucleotides from the 3′ ends of the viral cDNA in a reaction termed 3′ end processing. Second, IN covalently joins the recessed 3′ hydroxyls to the host DNA in a single step transesterification reaction termed strand transfer. The two ends of the viral DNA are joined 4–6 bp apart on the host DNA. Repair by host proteins yields the integrated provirus flanked by duplications of host sequence [2].

A major advance in the study of integrationwas the structure of the full length prototype foamy virus (PFV) IN Ref. [3]. A second structure revealed that the functional integration

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complex, termed an intasome, is a tetramer of PFV IN complexed with viral DNA ends [4]. These images offer unique insights into the moment of intasome binding to target DNA, but they do not inform the dynamic search mechanism of retroviral intasomes. Single molecule total internal reflection fluorescence (TIRF) microscopy studies showed PFV intasomes search target DNA by 1 dimensional (D) rotation coupled diffusion [5]. However, TIRF studies were not able to exclude the possibility that PFV intasomes also search by 3D diffusion or intersegmental transfer (IT). Transcription factors and DNA repair factors have been shown to employ a combination of 1D diffusion with 3D diffusion or IT, which may be necessary for searching DNA in the context of chromatin.

Large scale sequencing of retroviral integration sites reveals that both chromatin elements and sequence play distinct roles in the integrase site preference [6–8]. A subtle sequence preference at the integration site varies for each retrovirus [9]. HIV-1 IN favors actively transcribed genes while PFV IN prefers promoters and CpG islands [6]. Host co-factors that direct integration from multiple retroviruses have been identified as chromatin interacting proteins, which may be the determining factor for chromatin targeting [10–13]. No host co-factor has been identified for PFV IN. HIV-1 IN requires the host co-factor LEDGF/p75 for efficient integration both *in vivo* and *in vitro* [6,14]. The integrase binding domain of LEDGF/p75 has been shown to stabilize the HIV IN intasome [14]. In contrast, PFV IN readily forms intasomes *in vitro* without a stabilizing co-factor [3,15].

While retroviruses display diversity in integration site preferences, they also appear to adopt varying IN multimers in intasomes. PFV intasomes are a tetramer of IN, but retroviral IN octamers and hexadecamers have been reported [4,16–18]. It is unknown if all retroviral intasomes will search target DNA by similar mechanisms. HIV-1 IN has previously been shown to commit to a target DNA early *in vitro* [19]. Addition of a second target DNA at any time following the start of the reaction did not yield integration to the second target. This data suggests that HIV-1 IN does not perform 3D diffusion or IT search of target DNA. The caveat to these experiments is that the HIV-1 IN co-factor LEDGF/p75 had not yet been identified and was not included in the experiments [20]. LEDGF/p75 appears to bind chromatin *in vivo* by 1D and 3D diffusion [21]. Whether the HIV-1 intasome search of chromatin is defined by LEDGF/p75 diffusion or the lack of diffusion by HIV-1 IN is unknown.

Here we explore the interactions of PFV IN with target DNA. We report the role of divalent cation on the integration activity of PFV IN. In contrast to HIV-1 IN, PFV IN was found to readily perform integration to a second DNA up to an hour into a reaction. Finally, the PFV IN preferred integration site sequence was found to neither enhance nor inhibit integration *in vitro*.

2. Materials and methods

2.1. Purification of PFV IN

All chemicals were of the highest grade (Sigma Aldrich). Recombinant PFV IN was purified as described [22,23].

2.2. PFV integration reactions

DNA oligomers were purchased from Integrated DNA Technologies. Preprocessed PFV viral donor DNA was KEY616 5′ ATTGTCATGGAATTTTGTATATTGAGTGGCGCCCGAACAG 3′ annealed to KEY675 5′ CTGTTCGGGCGCCACTCAATATACAAAATTCCATGACA 3′. Blunt PFV viral donor DNA was KEY616 annealed to KEY623 5′ CTGTTCGGGCGCCACTCAATATACAAAATTCCATGACAAT 3′. Cy5 fluorophore labeling was at the 5′ end of KEY675 or KEY623.

PFV integration reactions were performed in 10mM HEPES, pH 7.5,110mMNaCl, 4 μMZnCl_2, 5mMMgSO_4, and 10mMDTT, 0.5 μM PFV IN, 1 μM viral donor DNA, 50 ng target DNA plasmid in a final volume of 15 μL. Where indicated 5mM MgCl_2, 5mM MnCl_2, or 5mMCaCl_2 were substituted for MgSO_4. Blunt viral donor DNA was used except when indicated. All reagents except target DNA were combined in 14 μL volume and incubated on ice for 15 min. Target DNA was added, reactions were incubated at 37 °C for 90 min, and stopped by the addition of 0.5% SDS, 1 mg/ml proteinase K. Reactions were incubated at 37 °C for an additional 60 min. Reaction products were separated by 1% agarose in TAE with 0.1 μg/mL ethidium bromide. Gels were scanned by Typhoon 9410 variable mode fluorescent imager (GE Healthcare) for ethidium bromide and Cy5. Images were analyzed by ImageQuant (GE Healthcare). Data was analyzed by paired *t*-test (GraphPad Prism).

2.3. PFV preferred integration site sequence

The PFV preferred integration site GTGCTAGCAC was subcloned to pMP2 between *Sac*I and *Sph*I sites and to pcDNA3.1 between *Kpn*I and *Xba*I sites [4]. DNA oligomers KEY709 5′ CGTGCTAGCACTCGCGAGCATG 3′ and KEY710 5′ CTCGCGAGTGCTAGCACGAGCT 3′ were annealed and subcloned to pMP2. DNA oligomers KEY725 5′ CGTGCTAGCACATCGATT 3′ and KEY726 5′ CTAGAATCGATGTGCTAGCACGGTAC 3′ were annealed and subcloned to pcDNA 3.1. Plasmids were confirmed by sequencing (Genewiz). Plasmids were relaxed by incubation with Nt.*Bsp*QI (NEB) at 50 °C for 1 h. Nt.*Bsp*QI nicks pMP2 once and pcDNA3.1 three times, on the minus strand at 2582 and 2792 and the plus strand at 3461. Annealed DNA oligomers KEY725 and KEY726 were added to integration reactions.

3. Results

3.1. Divalent cation preference of PFV IN

All retroviral INs require a divalent cation at the active site to assemble and perform both 3′ end processing and strand transfer reactions [1]. Several previous studies of HIV-1 IN evaluated the enzymatic preference for divalent cation [24–27]. HIV-1 IN appears to show a strong preference for manganese during assembly onto the viral DNA ends [26]. In contrast, calcium allows assembly of HIV-1 IN with viral DNA, but does not allow catalysis [24]. Recombinant HIV-1 IN is markedly more enzymatically active in manganese compared to magnesium [27]. HIV-1 IN was reported to be incapable of 3′ processing in the presence of magnesium [19]. The effects of different divalent cations have not been reported for PFV IN.

PFV IN activity was assayed with supercoiled plasmid target DNA [28]. Recombinant PFV IN is added to a Cy5 fluorescently labeled DNA oligomer mimicking the end of the viral cDNA (Fig. 1A). After incubation on ice to allow assembly of intasomes, a target plasmid is added and the reactions are incubated at 37 °C. The reaction products are separated by agarose gel stained with ethidium bromide. The PFV IN integration products are largely concerted integration (CI) of two viral donor DNA oligomers to the plasmid (Fig. 1A). The CI products have the mobility of linearized plasmid. A second integration product occurs when only one viral DNA donor is joined to the target plasmid. In this half site integration (HSI) reaction, a nick is introduced at the site of strand transfer relaxing the supercoils. The HSI products have the mobility of relaxed circular plasmid. The agarose gels are imaged for ethidium bromide and Cy5 fluorescence allowing for identification and quantitation of all DNA forms, including unreacted and reaction products.

We compared PFV IN integration to a supercoiled plasmid DNA in the presence of magnesium, manganese, or calcium (Fig. 1). Published protocols for HIV-1 IN assays often employ $MgCl_2$, but PFV IN assays utilize $MgSO_4$ [19,28]. Both divalent cations were assayed. Using a preprocessed viral DNA donor with recessed 3′ ends, this integration assay does not distinguish between assembly or strand transfer. There was little difference in the accumulation of CI products when either magnesium or manganese was present ($p > 0.05$). However, CI products in the presence of calcium were reduced to 22% of products observed in the presence of $MgSO_4$ ($p = 0.013$). HSI products were also assayed but showed little difference between the divalent cations assayed. The activity of PFV IN in the presence of calcium suggests that this enzyme is more permissive than HIV-1 IN, which has no enzymatic activity in calcium [24]. PFV IN favored CI to HSI in magnesium or manganese, but was more prone to HSI in calcium (HSI $MgSO_4$ compared to $CaCl_2$ $p = 0.036$).

PFV integration was also assayed with a blunt viral donor DNA (Fig. 1). This substrate requires PFV IN to perform 3′ end processing prior to strand transfer. These results showed greater differences between the divalent cations than preprocessed viral donor DNA. HSI products were similar in the presence of $MgSO_4$ or $MgCl_2$ ($p = 0.059$), but CI products increased by 30% in the presence of $MgCl_2$ ($p= 0.036$). PFV IN was more active in the presence of the manganese cation showing a 2.1 fold and 3.2 fold increase of CI ($p = 0.017$) or HSI ($p = 0.006$) products, respectively, compared to $MgSO_4$. PFV IN had no activity in the presence of calcium suggesting that 3′ end processing could not occur. Thus PFV IN may utilize calcium for assembly and strand transfer, but not 3′ end processing. Since PFV IN is functional in magnesium and this divalent cation is more physiologically relevant than manganese, subsequent experiments were performed with magnesium.

3.2. Target commitment

Real time single molecule experiments show that PFV intasomes may search over 1 kb of linear DNA [5]. It is unknown if a PFV intasome can switch targets by 3D diffusion following an unproductive search. Previous studies of HIV-1 IN suggested that this enzyme commits to a target DNA *in vitro* early and does not switch targets [19]. We tested the commitment of PFV IN to target DNA (Fig. 2). Integration reactions included two supercoiled plasmids, pMP2 and pcDNA 3.1, readily distinguished by agarose gel mobility.

The integration reactions were initiated with one plasmid at 37 °C. At variable times, a second plasmid was added to the reactions. Reciprocal reactions were performed, switching the order of the plasmids added.

PFV integration reactions revealed that simultaneous addition of two plasmids results in integration to both (Fig. 2). CI to the first plasmid in the reaction increased slightly throughout the time course. This observation was true whether 2858 bp pMP2 or 5397 bp pcDNA3.1 was the first plasmid added, but this difference was not statistically significant for either plasmid. CI to the second plasmid added to the reaction steadily decreased as the time of addition increased (simultaneous addition compared to 60 min pcDNA added second $p = 0.025$, pMP2 added second $p = 0.039$). HSI products were also quantified in these reactions and displayed similar trends to the CI products (simultaneous addition compared to 60 min pcDNA added second $p = 0.012$, pMP2 added second $p = 0.016$). Even when reactions had been incubated for 60 min, integration to the second plasmid is still readily detected. This suggests that a significant fraction of PFV IN does not commit to a target DNA within 60 min in stark contrast to HIV-1 IN.

3.3. Integration site sequence preference effects on integration

A unique yet subtle sequence preference for integration sites has been reported for different retroviruses [9]. Since PFV intasomes interact with naked DNA by 1D rotation coupled diffusion similar to lac repressor search for a sequence specific site, we tested the effect of the adding the PFV IN preferred integration site GTGCTAGCAC into target DNA [4,29]. The preferred integration site was subcloned into two different plasmids yielding pMP2-PFV or pcDNA3.1-PFV. PFV integration was compared between the parent plasmids and the preferred site plasmids (Fig. 3). Both blunt and preprocessed viral oligomer donors were tested in the assay. There were no apparent differences in the accumulation of integration products with the presence of the preferred sequence ($p > 0.05$).

Retroviral INs may have greater preference for structural features, particularly bent DNA, than sequence [30]. IN is known to favor the bent structure of supercoiled DNA compared to nicked, relaxed circles or linear DNA [5]. To evaluate integration efficiency with the sequence preference in the absence of supercoils, the plasmids were relaxed with a nicking endonuclease. In this context, there was no change in integration efficiency to the plasmids with the preferred sequence. These data suggest that the PFV IN preferred integration site does not enhance PFV integration.

Although the integration sequence preference did not enhance integration efficiency in target DNAs, excess small double stranded DNA oligomers were tested as competitors of integration. Double stranded DNA oligomers encoding the PFV preferred integration site sequence were titrated into integration assays (Fig. 4). The concentration of PFV IN is 500 nM, suggesting that the maximal concentration of tetrameric PFV intasomes in these reactions is 125 nM. The dsDNA oligomers were added at 5, 50, 500, or 5000M excess to monomeric PFV IN. PFV CI and HSI were unaffected by the addition of dsDNA oligomers at any concentration ($p > 0.05$). Taken together, the preferred sequence of PFV IN does not enhance or inhibit integration. It seems that structural elements are more important than sequence for integration targeting.

4. Discussion

Extensive mapping of integration sites has revealed that retroviral INs have unique preferences for sequence and chromatin features [6–8]. Bulk biochemical assays indicate that integrases prefer bent DNA [30]. Genomic DNA in the context of chromatin is the natural substrate for retroviral integrases making bent DNA an obvious target. However, even in the context of nucleosomes, integration does not appear to be random. A cryo-EM structure of the PFV intasome bound to a stable nucleosome revealed a single binding site [31]. Previous studies of IN with nucleosome substrates suggest that not all exposed DNA major grooves serve as targets for integrases [32].

Here we explore the dynamics of retroviral integrase interaction with target DNA. Intasomes may search for a target site by a variety of mechanisms including 1D diffusion (sliding), 3D diffusion (jumping), or IT. We have previously shown that PFV intasomes readily slide on DNA, but could not discern 3D diffusion or IT [5]. In this study, PFV IN is able to integrate to a second target DNA plasmid after an hour. While this data does not prove PFV IN performs 3D diffusion or IT, it is suggestive of these mechanisms. The PFV IN observations are in contrast to HIV-1 IN which did not integrate to a second DNA after 1 min and also appeared incapable of sliding on target DNA [19]. The HIV-1 IN data argues that this protein does not search by 3D diffusion nor IT. During an HIV-1 infection, the movement of HIV-1 integration complexes may be dictated by LEDGF/p75, which has been shown to search chromatin by at least 3D diffusion and likely IT [21]. PFV IN does not have a host co-factor and displays far greater mobility than reported for HIV-1 IN. Further experiments will be necessary to prove PFV IN 3D diffusion and/or IT searching. Similarly more sophisticated analysis, such as single molecule TIRF, may reveal that HIV-1 intasomes are capable of a more comprehensive search of target DNA than previously reported.

Each retrovirus has a subtle sequence preference at the integration site, only revealed after sequencing hundreds of integration sites [9]. Throughout the integration sequence preference, each base preference is independent of the preference for surrounding bases. Thus there is no interdependent relationship between base choice throughout the integration site preference. We explored the ability of the PFV preferred integration site sequence to either enhance or hinder integration. Adding the PFV integration site preference to two different plasmids did not increase the accumulation of integration products. Considering that DNA structure might be more important than sequence preference, the plasmids were relaxed with a nicking endonuclease to remove the preferred supercoils. Presence of the integration sequence preference in relaxed plasmids also did not increase integration product accumulation. These data suggested that the preferred sequence at PFV integration sites does not enhance integration to a naked DNA target plasmid *in vitro*.

Double stranded DNA oligomers encoding the PFV integration sequence preference were added to integration reactions to possibly inhibit integration. Such DNA oligomers were bound by the PFV intasome in a structure [4]. Even at large molar excess of the DNA oligomers, the accumulation of CI and HSI products were unaffected. The importance of retroviral integration site sequence preference appears to be minimal during integration *in vitro*.

Acknowledgments

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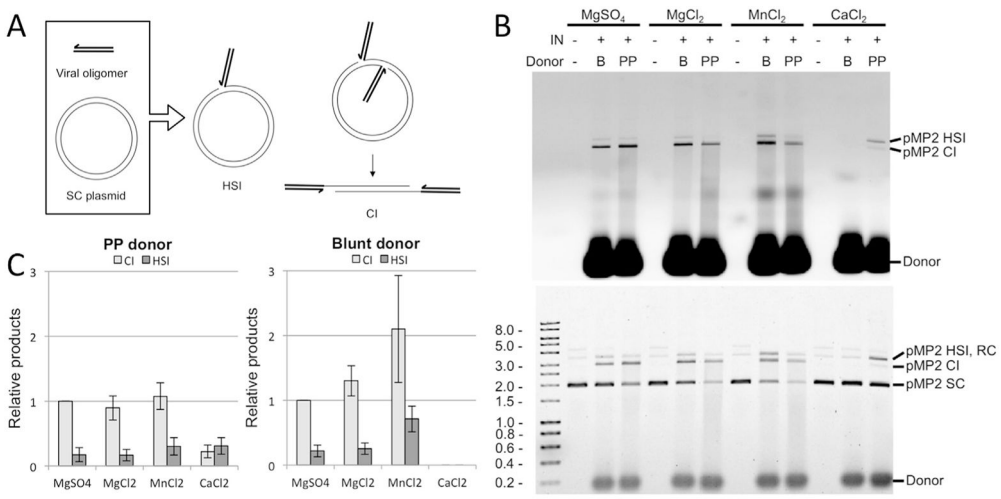

Fig. 1. PFV IN requirements for divalent cations

A) PFV IN with a viral donor DNA oligomer mimicking the ends of the viral cDNA is added to a supercoiled plasmid DNA to assay integration *in vitro*. The products may be half site integration (HSI) events where only one viral donor DNA is covalently joined to the target DNA. Integration introduces a nick in the plasmid and relaxation. Concerted integration (CI) is the joining of two viral donor DNAs to the plasmid. The product is a linearized plasmid. B) Agarose gel analysis of integration products generated in the presence of $MgSO_4$, $MgCl_2$, $MnCl_2$, or $CaCl_2$. Integration reactions employed Cy5 fluorescently labeled blunt donor DNA (B) or preprocessed donor DNA (PP). Target DNA is a 2.86 kb plasmid pMP2. Top panel, Cy5 image. Bottom panel, ethidium bromide image. Relaxed circles (RC) and supercoiled (SC) plasmid mobility is indicated. C) CI and HSI products were quantified from the Cy5 fluorescent images of agarose gels and expressed as relative to the CI product in $MgSO_4$. Error bars indicate the standard deviation between at least three independent experiments. DNA marker is kb.

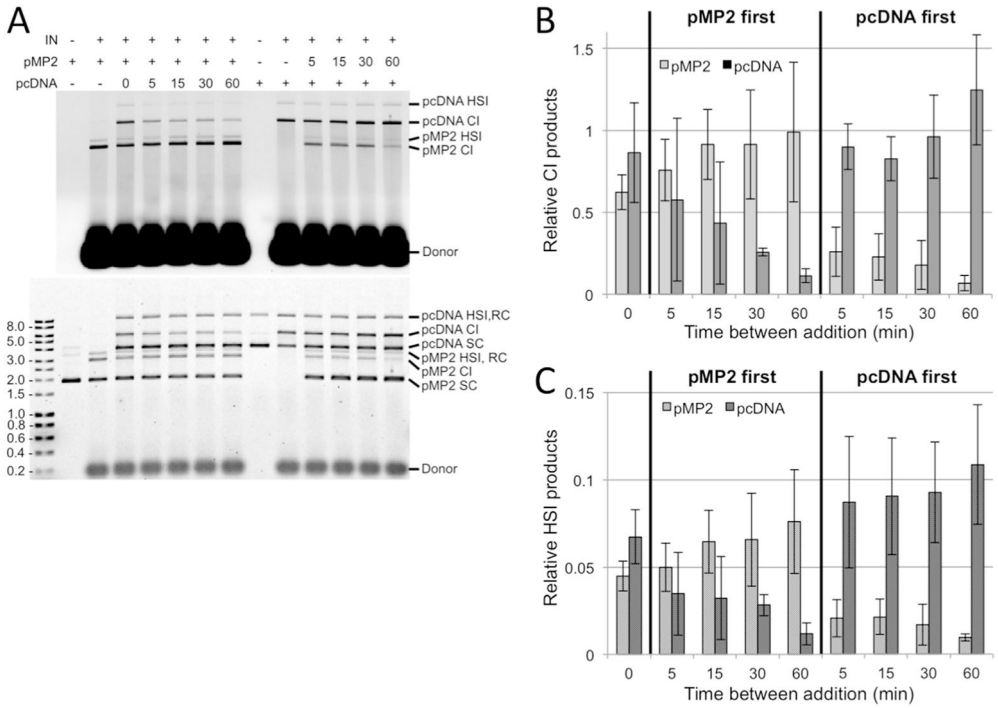

Fig. 2. PFV IN commitment to target DNA

A) PFV IN and Cy5-labeled viral donor DNA were added to 2.9 kb plasmid pMP2 or 5.4 kb plasmid pcDNA, indicated by + symbols. The second plasmid was added 5–60 min after the start of the reaction, indicated by numbers. Simultaneous addition of the two plasmids at the start of the reaction is indicated by 0. Integration reactions were a total of 90 min from the addition of the first plasmid. Reaction products were separated by agarose gel, stained with ethidium bromide, and scanned for Cy5 (top gel image) and ethidium bromide (bottom gel image). B) CI and C) HSI integration products were quantified and are expressed relative to the total integration observed in the single plasmid reaction. Error bars indicate the standard deviation between at least three independent experiments. DNA marker is kb.

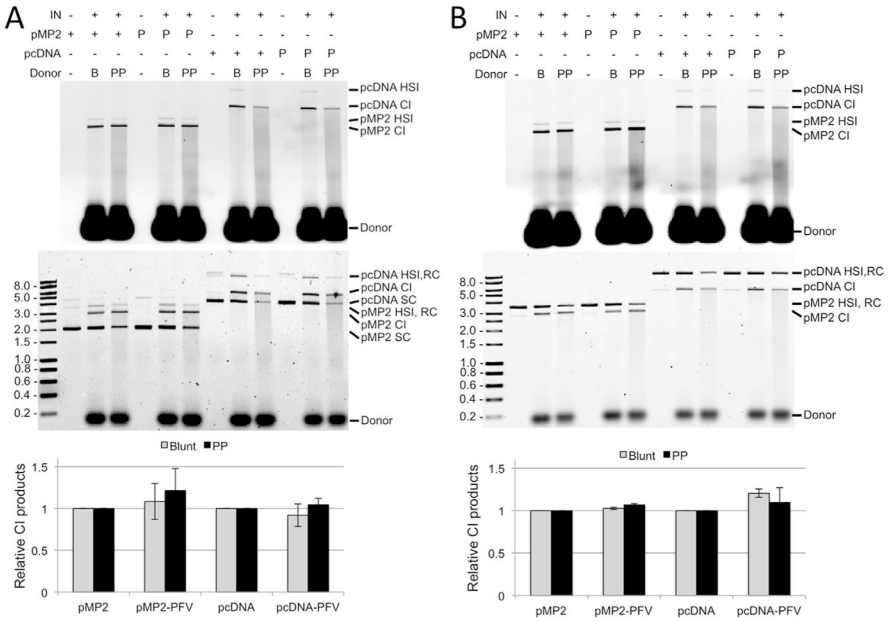

Fig. 3. PFV integration to plasmids encoding the preferred integration site

PFV IN and Cy5-labeled viral donor DNA were added to pMP2, pcDNA, and the parent plasmids with the 11 bp PFV IN preferred sequence, pMP2-PFV and pcDNA-PFV, indicated by P. The viral donor DNA was either blunt (B) or preprocessed (PP). The integration reaction products were analyzed by agarose gel. The Cy5 (top) and ethidium bromide (bottom) gel images are shown. The accumulation of integration products with pMP2-PFV and pcDNA-PFV are shown relative to pMP2 and pcDNA, respectively. A) PFV integration to supercoiled plasmids. B) PFV integration to relaxed plasmids. Error bars indicate the standard deviation between at least three independent experiments. DNA marker is kb.

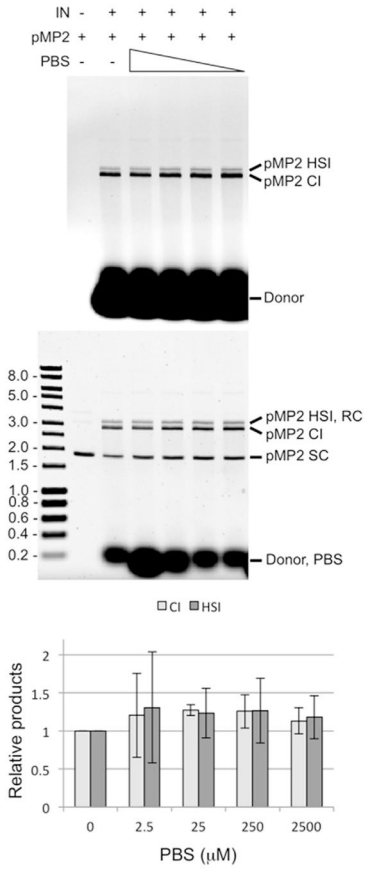

Fig. 4. PFV integration in the presence of DNA oligomers encoding the preferred integration site

PFV integration to plasmid pMP2 was performed in the presence of increasing concentrations of double stranded DNA oligomers encoding the PFV preferred integration site (PBS). Cy5 (top) and ethidium bromide gel images (bottom) are shown. Quantitation of the CI and HSI integration products are shown relative to the absence of integration site DNA oligomers. Error bars indicate the standard deviation between at least two independent experiments. DNA marker is kb.