



Short communication

Removal of nuclease contamination during purification of recombinant prototype foamy virus integrase



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A B S T R A C T

Retroviral infection requires integration of the viral genome into the host genome. Recombinant integrase proteins may be purified following bacterial expression. A bulk biochemical assay of integrase function relies on the conversion of supercoiled plasmids to linear or relaxed circles. Single molecule molecular tweezer assays of integrase also evaluate the conversion of supercoiled DNA to nicked and broken species. A bacterial nuclease that co-purifies with retroviral integrase may affect the quantitation of integration activity in bulk or single molecule assays. During purification of retroviral integrase from bacteria, fractions may be screened for contaminating nuclease activity. In order to efficiently separate the nuclease from integrase, the binding affinities of each protein must differ. We find that a co-purifying nuclease may be efficiently separated from integrase based on heparin affinity, but not ionic affinity.

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Article history:

Received 19 April 2016

Received in revised form 31 May 2016

Accepted 4 June 2016

Available online 5 June 2016

Keywords:

Retrovirus

Integrase

Protein purification

Retroviruses must integrate a copy of their viral genome to the host genome to continue the viral life cycle. Integration is performed by the viral enzyme integrase (Coffin et al., 1997). Recombinant integrase (IN) from many retroviruses may be purified following expression in bacteria (Jonsson et al., 1993; Marczinovits et al., 1992; Roth et al., 1988; Sherman and Fyfe, 1990; Terry et al., 1988; Valkov et al., 2009). After reverse transcription of the viral genomic RNA into a cDNA, retroviral IN has two catalytic activities *in vivo*. First, IN cleaves two nucleotides from each end of the viral cDNA resulting in recessed 3' hydroxyl groups, termed 3' processing. Second, IN joins each 3' hydroxyl of the viral cDNA to the host genomic DNA in a single step transesterification reaction, called strand transfer. The ultimate product of integration *in vivo* is the viral genome stably inserted to the host genome.

The preferences for retroviral integration sites *in vivo*, including chromatin features and subtle sequence preference, appear to be unique to each retrovirus (Holman and Coffin, 2005; Shun et al., 2007; Wu et al., 2005). These preferences continue to be explored and defined by assays *in vivo* and *in vitro* (Bennett et al., 2014; Hacker et al., 2006; Kang et al., 2006; Serrao et al., 2015; Taganov et al., 2004; Vora and Grandgenett, 1995). Bulk biochemical assays for integration activity *in vitro* utilize purified recombinant IN with two viral oligomer donor DNAs and a target supercoiled plasmid

(Cherepanov, 2007; Li and Craigie, 2005; Li et al., 2006; Sinha and Grandgenett, 2005; Valkov et al., 2009) (Fig. 1). Integration assay products are separated by agarose gel electrophoresis, which readily separates the relaxed circle, linear, and supercoiled species of a plasmid (Fig. 1B, lanes 2–4). IN mediated strand transfer of both viral DNA ends to the target plasmid DNA results in a linear product (Fig. 1A). Retroviral integration assays *in vitro* may also result in a non-physiologically relevant product consisting of a single viral DNA end joined to the target, termed half site integration (Goodarzi et al., 1995; Vora and Grandgenett, 1995). The half site integration product is a relaxed circle with a single viral DNA oligomer, a tagged circle. Quantitation of linear and relaxed products indicates concerted and half site integration, respectively.

The prototype foamy virus integrase (PFV IN) has become a model for studying integrase interactions with target DNA. PFV IN is sensitive to clinically relevant HIV-1 IN strand transfer inhibitors (Valkov et al., 2009). It was the first full length integrase protein to be crystallized with viral and target DNAs (Hare et al., 2010; Maertens et al., 2010). A recent cryo-EM structure illuminates the binding of a tetramer of PFV IN to a mononucleosome (Maskell et al., 2015). Finally, the first single molecule observations of retroviral IN complexes searching target DNA in real time utilized PFV IN (Jones et al., 2016). This protein has become arguably the most biophysically well-characterized retroviral IN.

DNA nucleases with non-specific nicking activity may also relax or linearize a supercoiled plasmid target DNA. The gel mobilities of plasmids relaxed or linearized by a co-purifying nuclease are indistinguishable from half site or concerted integration products,

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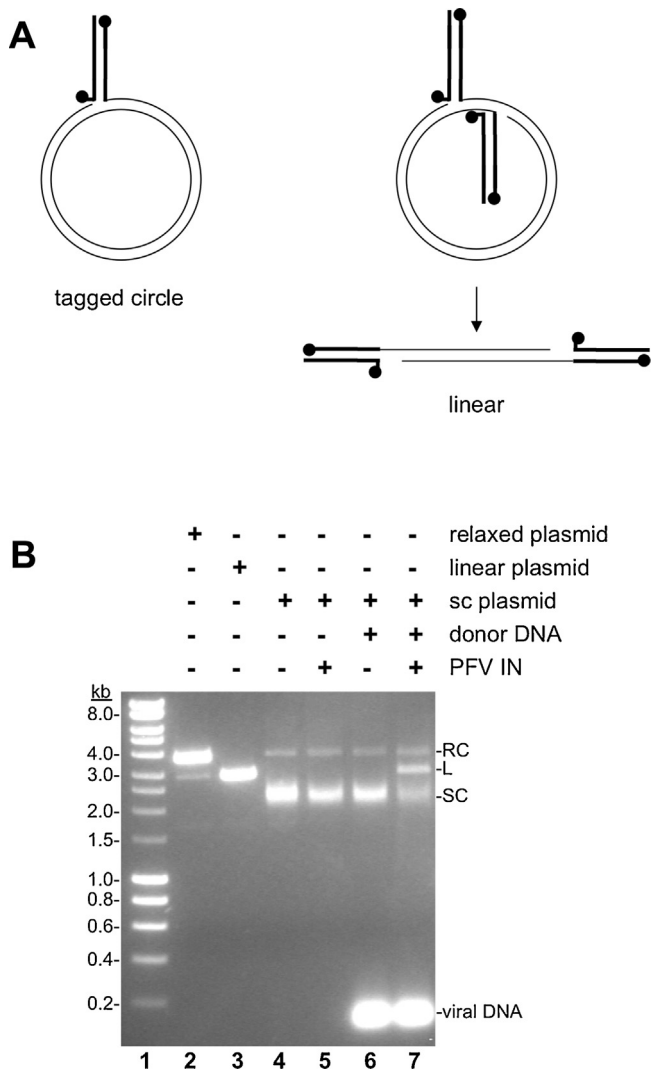


Fig. 1. PFV integration assay.

A. Cartoon of a bulk biochemical retroviral integration assay. Short oligomers of PFV viral DNA ends are shown as bold lines with balls indicating the 5' ends. Target DNA is shown as thin lines. Right, A supercoiled plasmid target DNA is relaxed by half site integration with a single viral donor DNA yielding a tagged circle. Half site integration is not physiologically relevant. Left, Alternatively, the supercoiled plasmid may be linearized by concerted integration with two viral donor DNA oligonucleotides. Concerted integration is the physiologically relevant integration reaction. B. Agarose gel electrophoresis separates the products of a PFV integration assay. The table at the top of the gel image indicates the addition of DNA forms and PFV IN. A supercoiled plasmid was treated with single strand nicking restriction nuclease Nt.BspQ1 which produces relaxed circles and minimal linear products (lane 2; RC, relaxed circle), linearized with BamHI (lane 3; L, linear), or untreated (lane 4; SC, supercoiled). Addition of PFV IN (lane 5) or viral donor DNA (lane 6) does not change the supercoiled plasmid. However, addition of PFV IN and viral donor DNA results in linear concerted integration products (lane 7).

respectively. Furthermore, supercoiled plasmid DNA is a preferred target for PFV IN compared to relaxed circles or linear DNA (Jones et al., 2016). A co-purifying nuclease that converts the plasmid target to relaxed or linearized DNAs will reduce the concentration of preferred supercoiled plasmid and alter the dynamics of the reaction. Thus a co-purifying bacterial nuclease may confound accurate quantitation of retroviral integration assay products *in vitro*.

During purification of recombinant PFV IN from *E. coli* strain BL21/DE3 Rosetta cells (Novagen), we noticed a non-specific nuclease activity. We used several chromatography affinity resins to characterize the contaminating bacterial nuclease that co-purifies with PFV IN. Size exclusion chromatography may be used to remove

possible co-purifying bacterial nucleases (Hare et al., 2010). However, the volume that may be loaded to size exclusion columns is typically limited, while affinity chromatography has no load volume limitations. There have been no previous reports of the binding affinities of the nuclease that co-purifies with retroviral IN. The differences in binding affinities of PFV IN and the nuclease suggest heparin affinity purification as an alternative strategy to size exclusion chromatography.

All retroviral INs possess inherent endonuclease activity for 3' processing and utilize a catalytic DDE motif (Coffin et al., 1997; Kulkosky et al., 1992; Sherman and Fyfe, 1990). We purified a catalytically inactive mutant PFV IN(D128N) to analyze and differentiate the binding affinities of IN and the co-purifying nuclease. PFV IN(D128N) and the nuclease have differing affinity for heparin sepharose, and Mono-S cation resin, but not Mono-Q anion resin. Purification and nuclease activity assays were performed three times with equivalent results; representative gels are shown here.

For purification from bacterial expression systems, retroviral IN expression constructs commonly include a hexa-histidine tag for initial purification with nickel resin (Cherepanov et al., 1999; Valkov et al., 2009; Villanueva et al., 2003). PFV IN(D128N) with an amino-terminal hexa-histidine tag was expressed in the Rosetta derivative of BL21(DE3) pLysS cells (Novagen). The culture was grown to OD₆₀₀ 0.9–1.0 and induced with 0.25 mM IPTG and 50 μM ZnCl₂ (all chemicals from Sigma-Aldrich) at 25° C for 4 h (Valkov et al.). The bacterial cells were sonicated in 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, and protease inhibitor PMSF. The lysate was centrifuged at 120,000g for 1 h at 4° C. The supernatant was separated by Ni-NTA Superflow resin (Qiagen) with a linear gradient of 20–200 mM imidazole, pH 8.0. Fractions were analyzed by PAGE stained with Coomassie Brilliant Blue. Fractions containing PFV IN(D128N) were combined and supplemented with 10 mM DTT and 0.1 mM EDTA. Tags, such as the hexa-histidine tag, may impair the ability of retroviral IN to form a functional multimer. A PreScission site between the tag and PFV IN allowed the hexa-histidine tag to be removed by digestion with human rhinovirus (HRV) 3C protease (Novagen) overnight at 4° C.

Recombinant retroviral IN is commonly fractionated by heparin sepharose affinity resin with a salt gradient following nickel chromatography (Cherepanov et al., 1999; Valkov et al., 2009; Villanueva et al., 2003). The digestion reaction was diluted with 3 vol 50 mM Tris-HCl, pH 7.5, 10 mM DTT, and 0.1 mM EDTA to reduce the salt concentration. Digested and diluted PFV IN(D128N) was bound to heparin sepharose resin in 50 mM Tris, pH 7.5, 10 mM DTT, 0.1 mM EDTA and eluted with a linear gradient of 0.2–1 M NaCl (Fig. 2A). Heparin fractions were analyzed by PAGE for the presence of IN protein (Fig. 2B) and by agarose gel electrophoresis for nuclease activity (Fig. 2C and D). Nuclease assays combined 3 μl of heparin fraction, 50 ng supercoiled plasmid pMP2, 10 mM HEPES, pH 7.5, 110 mM NaCl, 5 mM MgSO₄, 4 μM ZnCl₂, and 10 mM DTT in a final volume of 15 μl. No viral donor DNA was included in the nuclease assays. The reactions were incubated at 37° C for 90 min. Proteinase K and SDS were added to stop the reactions and digest proteins. DNA species were evaluated by agarose gel electrophoresis. Ethidium bromide stained gels were scanned with a Typhoon 9410 variable mode imager (GE Healthcare Life Sciences). Bands in each lane were quantified with ImageQuant TL software. The volume values of the relaxed circle, linear, and supercoiled bands were added to yield the total DNA volume in each lane. The percentage of the total DNA volume converted to relaxed circle and linear products is shown as “% Converted”. Reactions that did not include IN protein (No IN) displayed 4.4 (±0.7)% of the total DNA as relaxed circles or linear products. As a control wild type PFV IN was purified with Ni-NTA Superflow followed by heparin sepharose. The heparin sepharose fractions were analyzed by the endonuclease assay and

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