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Functional Nucleotides of U5 LTR Determining Substrate Specificity of Prototype Foamy Virus Integrase

Kang, Seung Yi[1], Dog Gn Ahn[1], Chan Lee[2], Yong Sup Lee[3], and Cha-Gyun Shin[1]*

[1]Department of Biotechnology, Chung-Ang University, Ansung 456-756, Korea
[2]Department of Food Science and Technology, Chung-Ang University, Ansung 456-756, Korea
[3]Kyung Hee East-West Pharmaceutical Research Institute, College of Pharmacy, Kyung Hee University, Seoul 130-701, Korea

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In order to study functional nucleotides in prototype foamy virus (PFV) DNA on specific recognition by PFV integrase (IN), we designed chimeric U5 long terminal repeat (LTR) DNA substrates by exchanging comparative sequences between human immunodeficiency virus type-1 (HIV-1) and PFV U5 LTRs, and investigated the 3'-end processing reactivity using HIV-1 and PFV INs, respectively. HIV-1 IN recognized the nucleotides present in the fifth and sixth positions at the 3'-end of the substrates more specifically than any other nucleotides in the viral DNA. However, PFV IN recognized the eighth and ninth nucleotides as distinctively as the fifth and sixth nucleotides in the reactions. In addition, none of the nucleotides present in the twelfth, sixteenth, seventeenth, eighteenth, nineteenth, and twentieth positions were not differentially recognized by HIV-1 and PFV INs, respectively. Therefore, our results suggest that the functional nucleotides that are specifically recognized by its own IN in the PFV U5 LTR are different from those in the HIV-1 U5 LTR in aspects of the positions and nucleotide sequences. Furthermore, it is proposed that the functional nucleotides related to the specific recognition by retroviral INs are present inside ten nucleotides from the 3'-end of the U5 LTR.

Keywords: Integrase, foamy, 3'-end processing, retroviral, U5 LTR

In the retroviral life cycle, the viral cDNA is incorporated into cellular DNA of the infected cells. This event is mediated by the virally encoded protein integrase (IN), and is termed integration, which is a highly ordered three-step process similar to reactions mediated by other members of the family of polynucleotidyl transferases [19, 26, 24]. In the first step of 3'-end processing, IN cleaves off the two terminal nucleotides at each 3'-end of the linear viral DNA, exposing a highly conserved CA dinucleotide on both strands [3, 10, 13]. The next step, called strand transfer or 3'-end joining, is a concerted cleavage-ligation reaction during which IN makes a staggered cut in the target DNA and ligates the recessed 3'-ends of the viral DNA to the 5'-ends of the target DNA at the cleavage site [9, 11]. The final step, 5'-end joining, resolves the gapped intermediate to the intact double-strand DNA in which cellular repair enzymes seal gaps on both strands [6].

In the integration process, the long terminal repeat (LTR) sequence as viral DNA ends is the only viral DNA region that is required for recognition by retroviral IN. The LTR sequences of various retroviruses have been shown to be necessary and sufficient for correct integration of viral DNA both *in vitro* and *in vivo* [4, 21, 27]. The subterminal dinucleotide, CA, located at the viral DNA end is absolutely required for integration in all retroviral DNAs. The sequences internal to the CA dinucleotide also appear to be required for optimal IN activity. However, the sequences are different from each other in retroviral species.

Foamy viruses (FVs), also called spumaviruses, are members of the retroviral family *Retroviridae*. The best-known FV is prototype foamy virus (PFV), previously referred to as human foamy virus (HFV), and PFV was initially isolated from lymphoblastoid cells of a Kenyan patient with a nasopharyngeal carcinoma [1]. Recent studies indicate that FVs are unconventional retroviruses and their particles have large amounts of functionally relevant DNA [17, 20].

Retroviral IN has viral DNA specificity in catalytic processes, since it recognizes its own viral DNA specifically by interacting with certain sequences of its own viral DNA ends. By using synthetic duplex oligonucleotide substrates that mimic the U5 or U3 termini of retroviral DNA, biochemical characteristics of oncoretroviral and lentiviral integration reactions *in vitro* have been well documented [8, 21, 30], whereas only a few studies on foamy viral IN

*Corresponding author
Phone: 82-31-670-3067; Fax: 82-31-675-0409;
E-mail: cgshin@cau.ac.kr

have been reported [22, 23]. We recently characterized the functional domains and residues in PFV IN [16]. However, functional nucleotide(s) of PFV DNA ends for specific integration mediated by PFV IN has yet to be demonstrated. Here, in order to study the critical nucleotide sequences determining the viral DNA specificity of PFV IN, we constructed chimeric U5 LTR substrates by exchanging the comparative nucleotides between human immunodeficiency virus type-1 (HIV-1) and PFV U5 LTRs, and investigated the reactivity to the chimeric U5 LTR substrates. Our results showed that PFV IN recognizes the eighth and ninth nucleotides as distinctively as the fifth and sixth nucleotides in the enzymatic reactions, whereas HIV IN recognizes the fifth and sixth nucleotides more distinctively.

MATERIALS AND METHODS

Construction of Expression Vectors

The expression vectors for the HIV-1 IN and the PFV IN were constructed by ligation of the DNA fragments amplified from the proviral DNA (HXBc2 and pHSRV) to the NdeI and BamHI sites of pET15b, as described previously [16]. The resultant recombinant constructs are characterized to contain six histidine codons in front of the integrase sequence. The presence of six histidines in the expressed protein provides a simple purification based on the selective affinity for a nickel-chelated absorbent [12, 14].

Expression and Purification of IN Proteins

The DNA constructs were transformed into *E. coli* BL21 (DE3). The cells were grown at 37°C in 2 l of LB medium containing 50 μg ampicillin/ml. At an optical density of 0.8, isopropyl-1-thio-β-D-galactopyranoside was added to 0.3 mM for expression induction, and the culture was grown for an additional 4 h [29]. After harvesting, the cell pellet was frozen at −80°C. Frozen bacterial pellets were thawed and resuspended in 64 ml of S1 lysis buffer [50 mM Tris·HCl (pH 7.6), 20 mM β-mecaptoethanol, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 10 mM imidazole]. The cell suspension was kept on ice for 30 min. Then, 16 ml of 5 M NaCl and 8.8 ml of 100 mM CHAPS were added. The suspension was sonicated for 3 min on ice and centrifuged at 100,000 ×*g* for 1 h at 4°C. The supernatant was directly loaded onto a column of nickel-chelated nitrilotriacetic acid agarose (bed volume of 1 ml, Qiagen) pre-equilibrated with S10 buffer (S1 buffer containing 1 M NaCl and 10 mM CHAPS). The resin was washed four times with 3 ml of S10 buffer. Protein was eluted eight times with 0.5 ml of S100 buffer (S1 buffer containing 1 M NaCl and 100 mM CHAPS). The fractions containing the protein were collected, dialyzed against S10 buffer, and stored at −80°C for further experiments. To remove the His tag, the isolated protein was incubated with bovine thrombin (Sigma; 25 NIH units/mg of integrase) at 30°C for 4 h. The sample treated with thrombin was then diluted with 9 volumes of S25 buffer [50 mM Tris·HCl (pH 7.6), 20 mM β-mecaptoethanol, 0.1 mM EDTA, 1 mM phenylmethylsulfony fluoride, 10% glycerol, 10 mM CHAPS, and 50 μM ZnCl] before loading onto a column containing 0.25 ml of SP-Sepharose (Pharmacia). The column was then washed with 4 ml of S25-100 buffer (S25 buffer containing 100 mM NaCl). The protein was four times eluted with 0.15 ml of S25-300 buffer (S25 buffer containing 300 mM NaCl) and then 0.15 ml of S25-600 buffer (S25 buffer containing 600 mM NaCl), respectively, and stored as aliquots at −80°C. Protein concentrations were determined by the Bradford method (Bio-Rad) using bovine serum albumin as a standard.

Preparation of Chimeric U5 LTR Substrates

To prepare chimeric substrates, terminal sequences of each viral U5 LTR were compared. The nucleotides of one viral U5 LTR were replaced with those of another viral U5 LTR at the positions where sequences are different from each other. The sequences of chimeric oligonucleotides used for enzymatic assays are summarized on the figures in the Results and Discussion section below.

3'-End Processing Cleavage Activities

The 3'-end processing activities were assayed as described previously [2]. The oligonucleotides were purified by electrophoresis through a 15% denaturing polyacrylamide gel. The 5'-end of (+) sense oligonucleotides was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and then annealed with their complementary oligonucleotides, respectively.

In all assays, unless indicated otherwise, 0.1 pmol of the DNA substrate was incubated with 2 pmol of integrase for 60 min at 37°C in 10 μl of reaction buffer containing a final concentration of 20 mM HEPES (pH 7.5) and 5 mM $MnCl_2$. The reaction was stopped by the addition of 18 mM EDTA, pH 8.0. The reaction products were mixed with an equal volume of loading buffer (98% deionized formamide, 10 mM EDTA, pH 8.0, 0.05% bromophenol blue, 0.05% xylene cyanol), and heated at 90°C for 3 min before analysis by electrophoresis on a 15% polyacrylamide gel with 7 M urea in Tris-borate EDTA buffer. Quantitation of the products was carried out with a Molecular Dynamics PhosphoImager (GS525, BioRad).

RESULTS AND DISCUSSION

Preparation of Chimeric U5 LTR Substrates

In order to prepare chimeric U5 LTR substrates, the twenty nucleotide sequences at the 3'-end of HIV-1 U5 LTR and PFV U5 LTR were compared. There were 11 positions whose sequences were different between the two U5 LTRs (indicated as italized below);

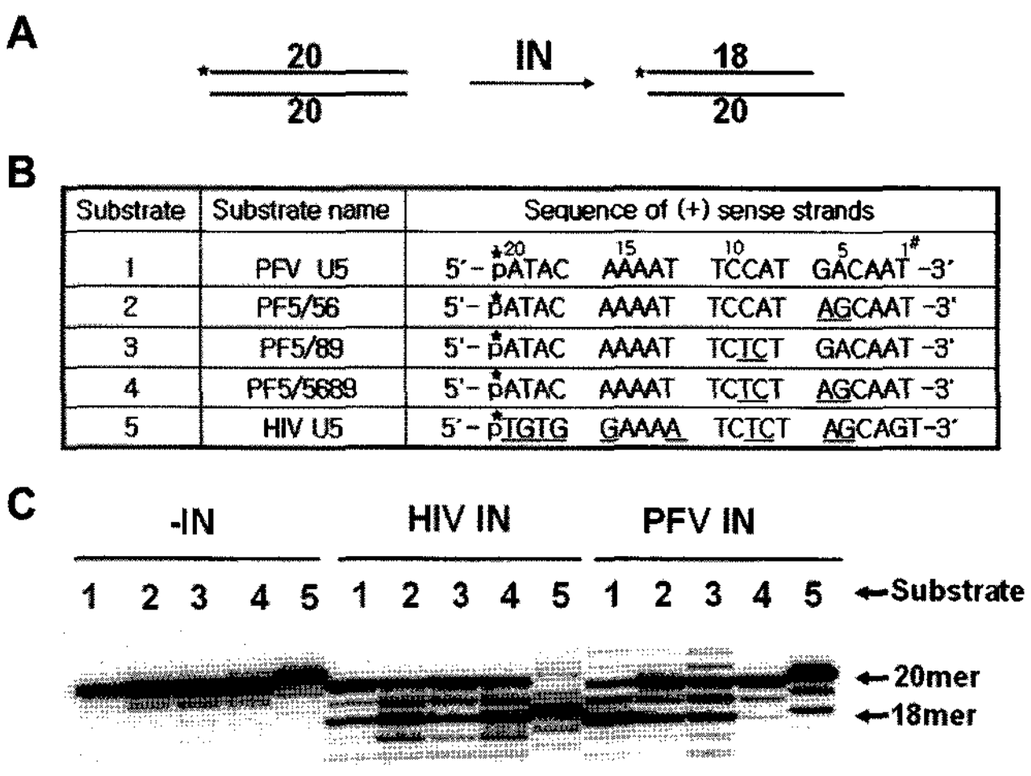
HIV-1 U5 LTR: 5'-*TGT* *GG* AAA*A* TC *TC* T *AG* **CA***G*T-3'
PFV U5 LTR: 5'-*ATA* *CA* AAA*T* TC *CA* T *GA* **CA***A*T-3'.

The HIV-1 and PFV INs cleave off the last two nucleotides (GT for HIV-1, AT for PFV) at the 3'-end of their U5 LTR DNA in the 3'-end processing reaction, respectively (Fig. 1A). The reactions can be evaluated by measuring conversion of the 20mer oligonucleotide to the 18mer oligonucleotide by using substrate radiolabeled at the 5'-end of the (+) sense strand of the duplex oligonucleotide substrates (Fig. 1A). The variation of sequences of the last two nucleotides was known to have negligible effect on the

enzymatic reactions [31]. The invariant sequence CA (indicated as bold above) among retroviral DNA is located at the third and fourth positions at the 3'-end of viral DNA. Earlier studies had shown that mutation of these nucleotides almost blocks 3'-end processing cleavage [25, 31]. Depending on the size and position, alteration in the sequences internal to the CA dinucleotide resulted in significant or negligible reduction of 3'-end processing cleavage [15]. However, there were no detailed investigations for functional nucleotides of PFV U5 LTR determining substrate specificity of PFV IN. Here, we construct various PFV chimeric U5 LTR substrates by introducing nucleotides of the HIV-1 U5 LTR at the comparative sites (marked as underlined above).

Nucleotides of Viral U5 LTR Determining Substrate Specificity of PFV IN

The LTR termini are the only viral sequences required *in cis* for recognition by the integration machinery [18, 27]. Therefore, it is thought that differences in sequences internal to the subterminal CA dinucleotide are associated with substrate specificity of retroviral IN. In order to find nucleotides determining the substrate specificity of HIV-1 and PFV INs, initially the 3'-end processing reactivities were tested by using the chimeric substrates that had been replaced with nucleotides of HIV-1 substrate at the corresponding sites within ten nucleotides from the 3'-end of the PFV U5 LTR, as shown in Fig. 1B. When HIV-1 IN was incubated with various substrates, HIV U5 (wild-type HIV-1 substrate) was well cleaved but PFV U5 (wild-type PFV substrate) was not (Fig. 1C; lanes 5 and 1 in the HIV IN group, respectively). The chimeric substrate, PF5/56, which has the same nucleotides as HIV U5 has at the fifth and sixth positions was more efficiently cleaved than the chimeric substrate, PF5/89, which has the same nucleotides as HIV U5 has at the eighth and ninth positions (Fig. 1C; lanes 2 and 3 in the HIV IN group, respectively). In addition, the chimeric substrate, PF5/5689, which has the same nucleotides as HIV U5 has at the fifth, sixth, eighth, and ninth positions, was well cleaved as PF5/56 was (Fig. 1C; lanes 2 and 4 in the HIV IN group, respectively). These results indicate that HIV-1 IN recognizes the fifth and sixth nucleotides more distinctively than the eighth and ninth nucleotides as a mark for its own substrate. On the other hand when PFV IN was incubated with the substrates, it cleaved the chimeric substrates, PF5/56 and PF5/89, to a similar extent (Fig. 1C; lanes 2 and 3 in the PFV IN group, respectively). Furthermore, it was not able to cleave the chimeric substrate HF5/5689 (Fig. 1C; lane 4 in the PFV IN group). Since replacement of either the fifth and sixth or the eighth and ninth nucleotides was able to reduce the cleavage of the substrate to a similar extent, the results indicate that PFV IN recognizes the fifth and sixth nucleotides and the eighth and ninth nucleotides at the same level as marks for its own DNA substrate.



Substrate	Substrate name	Sequence of (+) sense strands
1	PFV U5	5'-p*ATAC AAAAT TCCAT GACAAT-3'
2	PF5/56	5'-p*ATAC AAAAT TCCAT AGCAAT-3'
3	PF5/89	5'-p*ATAC AAAAT TCTCT GACAAT-3'
4	PF5/5689	5'-p*ATAC AAAAT TCTCT AGCAAT-3'
5	HIV U5	5'-p*TGTG GAAAA TCTCT AGCAGT-3'

Fig. 1. Differential responses of HIV-1 or PFV INs on the PFV chimeric U5 LTR substrates in the 3'-end processing reactions.
A. Schematic diagram of the *in vitro* 3'-end processing reaction. IN cleaves off two nucleotides at the 3'-end of the duplex oligonucleotide DNA mimicking the U5 LTR end. **B**. Sequence of (+) sense strands of PFV chimeric U5 LTR substrates. The nucleotides of the fifth and sixth positions and/or the eighth and ninth positions located from the 3'-end of the HIV-1 U5 LTR were introduced into the corresponding positions of the PFV U5 LTR. #: Numbers indicate positions of nucleotides distant from the 3'-end of the PFV or HIV-1 U5 LTR. **C**. 3'-End processing reactions. The DNA substrates were prepared by radiolabeling (★ in **B**) the (+) sense strand oligonucleotides (20mer), and by annealing with the complementary oligonucleotides (20mer), respectively. The labeled substrates of 0.1 pmol were incubated at 37°C for 60 min with purified IN (indicated above) of 2 pmol in 20 mM Hepes (pH7.5) and 5 mM $MnCl_2$. Conversion of the 20mer oligonucleotides to the 18mer oligonucleotides was analyzed in a 15% polyacrylamide gel. -IN, Substrate only; HIV IN, HIV-1 IN was added; PFV IN, PFV IN was added.

In order to investigate these results quantitatively, the 3'-end processing cleavage was tested in various reaction times such as 5, 15, 60, and 120 min. The results are summarized in Fig. 2. After 60 min incubation with HIV-1 IN, the cleavage reactions occurred in 16.5±4.5%, 66.7±6.4%, 22.7±7.3%, 67.6±8.2%, and 94.2±4.3% for PFV U5, PF5/56, PF5/89, PF5/5689, and HIV U5, respectively (Fig. 2A). The overall reaction profiles showed that HIV-1 IN cleaved PF5/56 more efficiently than PF5/89. In contrast, PFV IN cleaved the substrates in the 60 min reactions at the levels of 86.5±8.4%, 29.3±7.3%, 34.5±5.2%, 5.2±4.5%, and 8.6±6.7% for PFV U5, PF5/56, PF5/89, PF5/5689, and HIV U5, respectively (Fig. 2B). PF5/5689 was not cleaved well in all reaction times, whereas PF5/56 and PF5/89 were efficiently cleaved by PFV IN to similar extents, respectively.

As we were interested to confirm in reverse the conclusion derived from the results of Figs. 1 and 2, the HIV chimeric U5 LTR substrates that have nucleotides of PFV U5 LTR in the backbone of HIV U5 LTR were prepared (Fig. 3A) and tested for the 3'-end processing

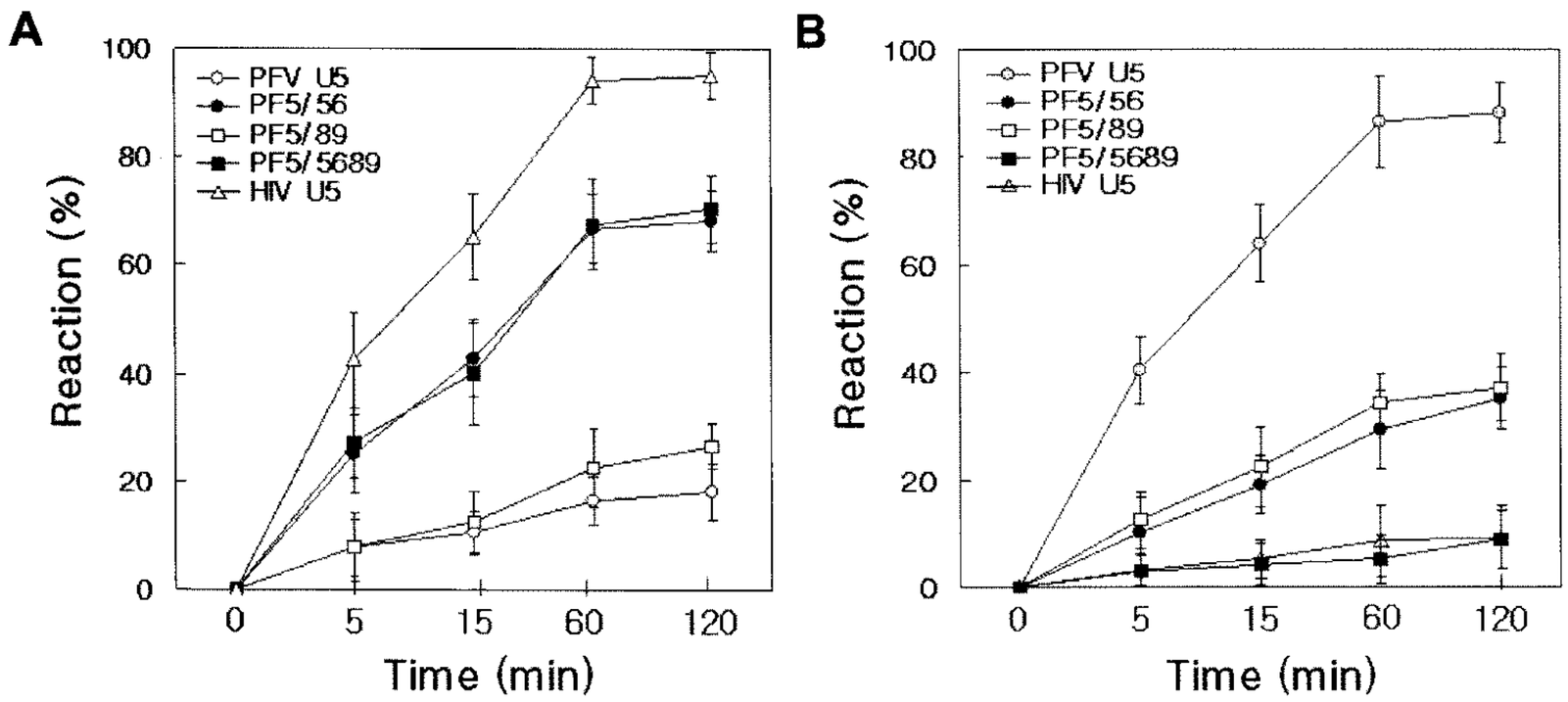


Fig. 2. Reaction time-dependent increase of 3'-end processing products.
The wild-type and PFV chimeric substrates were incubated with HIV-1 (**A**) or PFV IN (**B**) for 0, 5, 15, 60, and 120 min, respectively. Products were analyzed by electrophoresis, and the conversion of the 20mer to the 18mer was calculated with a phosphoimage analyzer. The percent reaction was determined as 100× 18mer/(18mer+20mer). The data represent the mean±SEM and are representative of three to four independent experiments.

cleavage. HIV-1 IN cleaved the substrates HIV U5, HI5/56, HI5/89, HI5/5689, and PFV U5 to 91.3±5.7%, 54.6±7.0%, 80.5±6.2%, 29.3±4.9%, and 14.5±6.7% in the 60 min reactions, respectively (Fig. 3B). The results showed that HIV-1 IN cleaved HI5/56 less efficiently than HI5/89, indicating that replacement of the fifth and sixth nucleotides in the wild-type HIV-1 substrate reduces reactivity of the substrate to the HIV-1 IN more effectively than replacement

A

Substrate	Substrate name	Sequence of (+) sense strands
1	HIV U5	5′ – ★pTGTG GAAAA TCTCT AGCAGT-3′ (20 15 10 5 1#)
2	HI5/56	5′ – ★pTGTG GAAAA TCTCT GACAGT -3′
3	HI5/89	5′ – ★pTGTG GAAAA TCCAT AGCAGT -3′
4	HI5/5689	5′ – ★pTGTG GAAAA TCCAT GACAGT -3′
5	PFV U5	5′ – ★pATAC AAAAT TCCAT GACAAT -3′

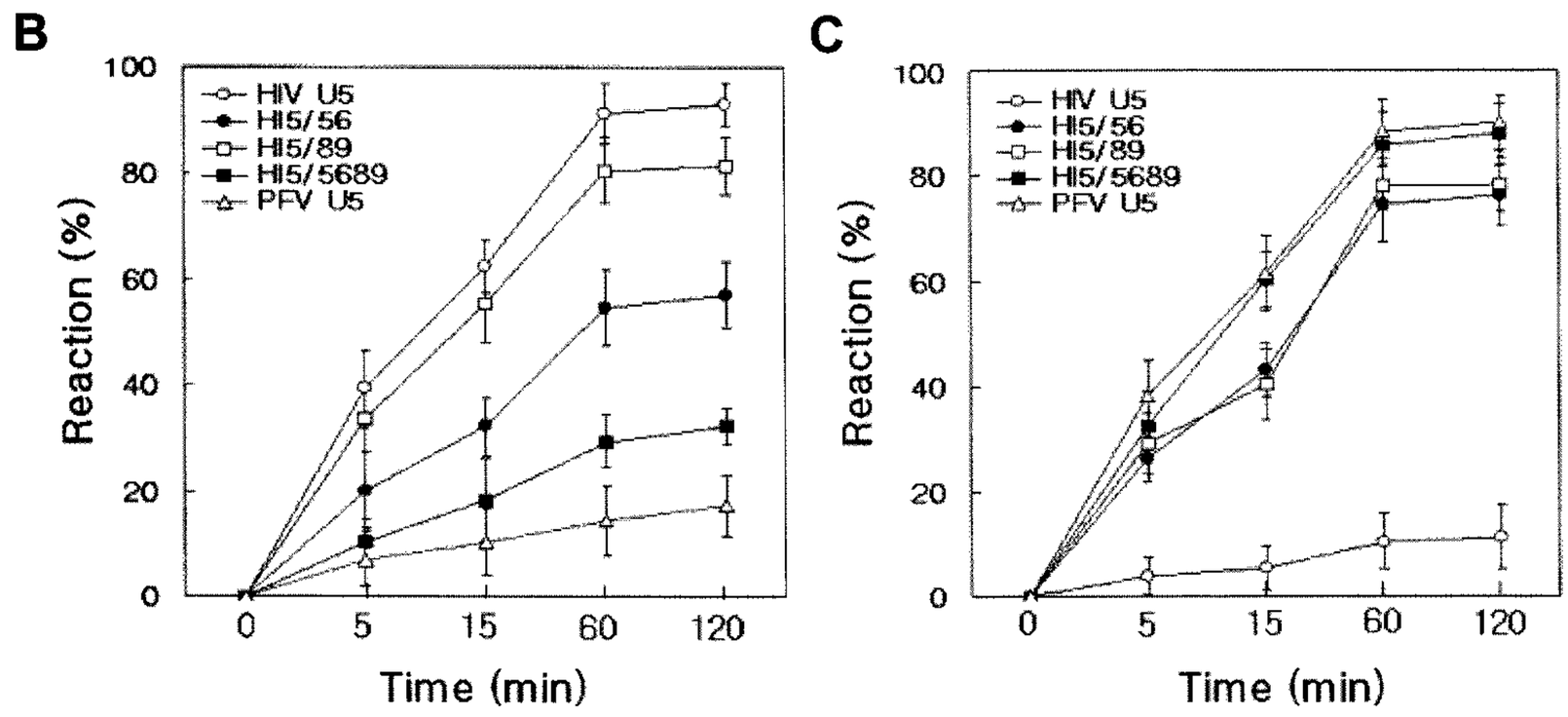


Fig. 3. 3'-End processing reactions using the HIV chimeric U5 LTR substrates.
A. Sequence of (+) sense strands of HIV-1 chimeric U5 LTR substrates. The nucleotides of the fifth and sixth positions and/or the eighth and ninth positions located from the 3'-end of the PFV U5 LTR were introduced into the corresponding positions of the HIV-1 U5 LTR. #: Numbers indicate positions of nucleotides distant from the 3'-end of the HIV-1 or PFV U5 LTR. ★: indicates radiolabeling of the phosphate at the 5'-end of the (+) sense strand of the substrates. **B**, **C**. 3'-End processing reactions using the HIV-1 chimeric U5 LTR substrates in the presence of HIV-1 IN (**B**) or PFV-IN (**C**). The wild-type and HIV chimeric substrates were incubated with HIV-1 or PFV IN for 0, 5, 15, 60, and 120 min, respectively. Products were analyzed by electrophoresis, and the conversion of the 20mer to the 18mer was quantitated with a phosphoimage analyzer. The percent reaction was determined as 100×18mer/(18mer+20mer). The data represent the mean±SEM and are representative of three to four independent experiments.

of the eighth and ninth nucleotides does. Same patterns of results were observed in the 5, 15, and 120 min reactions (Fig. 3B).

In contrast, PFV cleaved the substrates HIV U5, HI5/56, HI5/89, HI5/5689, and PFV U5 to 10.5±5.3%, 74.6±7.2%, 78.3±4.9%, 85.8±6.7%, and 88.5±6.0% in the 60 min reactions, respectively (Fig. 3C). PFV IN cleaved HI5/56 and HI5/89 to a similar level, but HI5/5689 more efficiently. Similar results were consistently observed in the 5, 15, and 120 min reactions. Therefore, these experiments support the previous conclusion that HIV-1 IN recognizes the fifth and sixth nucleotides more distinctively than the eighth and ninth nucleotides, whereas PFV IN recognizes the fifth and sixth nucleotides and the eighth and ninth nucleotides to a similar level.

To investigate whether or not a difference in the nucleotide sequence, present more than 10 nucleotides away from the 3'-end of the U5 LTR, influences the reactivity of HIV-1 and PFV INs, chimeric substrates were prepared by introducing nucleotide(s) of the other viral U5 LTR at the comparative site(s) (Fig. 4A). PF5/①, PF5/②, and PF5/③ have one to three nucleotide(s) of the HIV-1 U5 LTR at the twelfth, sixteenth, and seventeenth, and eighteenth, nineteenth, and twentieth position(s) in the HFV U5 LTR substrate backbone, respectively. In addition HI5/①, HI5/②, and HI5/③ have one to three nucleotide(s) of the PFV U5 LTR at the twelfth, sixteenth, and seventeenth, and eighteenth, nineteenth, and twentieth position(s) in the HIV-1 U5 LTR substrate backbone, respectively. The 3'-end cleavage results of the chimeric substrates are shown in Fig. 4B. HIV-1 IN hardly cleaved the chimeric PFV U5 LTR substrates (PF5/①, PF5/②, and PF5/③) and the wild-type PFV U5 LTR substrate (Fig. 4B, lanes 1 to 4 in the HIV IN group), but cleaved very efficiently the chimeric HIV-1 U5 LTR substrates and the wild-type HIV-1 U5 LTR substrate (Fig. 4B, lanes 5 to 8 in the HIV IN group). Similarly, PFV IN cleaved well the chimeric PFV U5 LTR substrates and the wild-type PFV U5 LTR substrate (Fig. 4B, lanes 1 to 4 in the PFV IN group), but hardly cleaved the chimeric HIV-1 U5 LTR substrates and the wild-type HIV-1 U5 LTR substrate (Fig. 4B, lanes 5 to 8 in the PFV IN group). It indicates that replacement of nucleotides at these sites did not influence the reactivity of HIV-1 and PFV INs to the substrates.

A

Substrate	Substrate name	Sequence of (+) sense strands
1	PFV U5	5′- p*ATAC AAAAT TCCAT GACAAT -3′
2	PF5/①	5′- p*ATAC AAAAA TCCAT GACAAT -3′
3	PF5/②	5′- p*ATAG GAAAT TCCAT GACAAT-3′
4	PF5/③	5′- p*TGTC AAAAT TCCAT GACAAT -3′
5	HIV U5	5′- p*TGTG GAAAA TCTCT AGCAGT-3′
6	HI5/①	5′- p*TGTG GAAAT TCTCT AGCAGT-3′
7	HI5/②	5′- p*TGTC AAAAA TCTCT AGCAGT-3′
8	HI5/③	5′- p*ATAG GAAAA TCTCT AGCAGT-3′

B

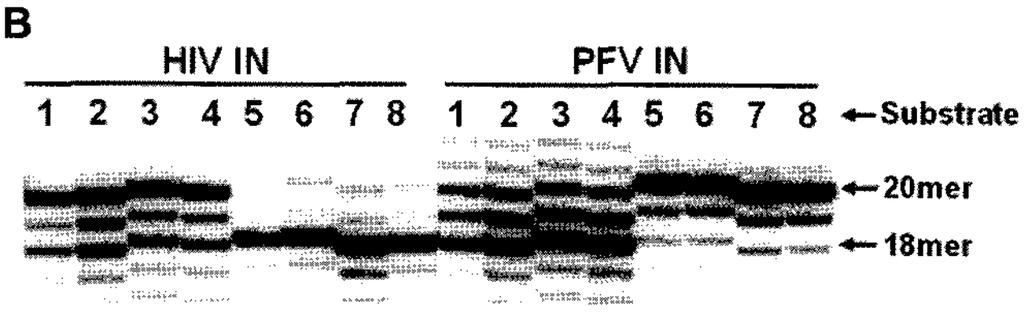


Fig. 4. Failure of differential responses of HIV-1 or PFV IN on the chimeric U5 LTR containing different nucleotides at the far internal sites.

A. Sequence of (+) sense strands of PFV or HIV-1 chimeric U5 LTR substrates. The nucleotides of the twelfth, sixteenth, seventeenth, eighteenth, nineteenth, and twentieth positions located from the 3'-end of the one viral U5 LTR were replaced with those of the corresponding positions of the other viral U5 LTR. **B**. 3'-End processing reactions of HIV-1 or PFV IN on the chimeric U5 LTR substrates. Subsequent procedures are same as Fig. 1.

Previously, it was suggested that the critical bases required for function of HIV-1 IN lies between positions 2 and 9 [5]. In addition, Masuda *et al.* [18] reported that terminal 11 base pairs of viral DNA are sufficient for specific recognition by HIV-1 IN. Therefore, our result suggesting that the nucleotides at the positions 5 and 6 are critical ones for substrate specificity of HIV-1 IN are consistent with the earlier works [5, 15, 18, 31]. However, there are distinctive properties between HIV-1 and PFV INs in recognizing their own substrate DNA, since the sequences and positions of the nucleotides critically involved in substrate recognition are different. Probably, differences in nucleotide sequences of the LTR end reflect differences in amino acid sequences of the active sites of retroviral INs [7].

With our results and others, therefore, it is suggested that retroviral IN recognizes distinctive nucleotides on its own substrate, which determines substrate specificity [25]. Although all nucleotides at the viral DNA ends are not absolutely required for specific IN activity, several nucleotides internal to the invariant CA at the viral DNA termini interact with retroviral IN, which contributes to specific recognition. In the case of PFV IN, the nucleotides present at the positions 5 and 6, and 8 and 9 of the U5 LTR end are recognized to a similar level as marks for specific recognition. This study is the first report to explain specific interaction of PFV IN with viral DNA. In addition, it will contribute to developing inhibitors against viral replication as targeting to viral integrase, as Snasel *et al.* [28] showed that HIV-1 IN is efficiently inhibited by modified oligonucleotides derived from U5 LTRs.

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