We constructed deletion mutants and seven point mutants by polymerase chain reaction to investigate the specificity of feline foamy virus integrase functional domains. Complementation reactions were performed for three enzymatic activities such as 3'-end processing, strand transfer, and disintegration. The complementation reactions with deletion mutants showed several activities for 3'-end processing and strand transfer. The conserved central domain and the combination of the N-terminal or C-terminal domains increased disintegration activity significantly. In the complementation reactions between deletion and point mutants, the combination between D107V and deletion mutants revealed 3'-end processing activities, but the combination with others did not have any activity, including strand transfer activities. Disintegration activity increased evenly, except the combination with glutamic acid 200. These results suggest that an intact central domain mediates enzymatic activities but fails to show these activities in the absence of the N-terminal or C-terminal domains. [BMB Reports 2013; 46(1): 53-58]

INTRODUCTION

Foamy viruses (FVs) belong to the family Retroviridae of the Spumaretrovirinae subfamily with only one genus (Spumavirus) (1). Spumaviruses are representative prototype foamy viruses (PFV) previously called human foamy virus and feline foamy virus (FFV). The foamy viruses (FVs) are endemic to most non-human primates (NPS), cats, cattle, and horses as well as humans who have acquired the infection from NPS (2, 3), which was first isolated in 1971 (4). FVs are also anticipated to be an available candidate as a gene therapy vector (5).

The virally encoded protein integrase (IN) is involved in the integration event, which is a highly ordered three-step process similar to reactions mediated by other members of the polynucleotidyl transferase family (6). Retroviral IN carries out of order DNA cleavage and joining reactions during viral integration, 3'-end processing (or endonucleolytic) activity, and strand transfer (integration or 3'-end joining) activity, respectively. In the first step of the 3'-end processing reaction, the highly conserved CA dinucleotide from the 3'-end of linear viral DNA exposes the 3'-hydroxyl group at both ends (7, 8). The second step, called the strand transfer reaction, is a concerted cleavage-ligation reaction during which IN makes a staggered cut in the chromosomal targeted DNA and ligates the recessed 3'-ends of the pre-processed viral DNA into opposing strands at the target DNA cleavage site (9, 10). The final step, called disintegration (or 5'-end joining), resolves the gapped intermediate of the intact double-stranded DNA, which is repaired by host cell enzymes to produce a stable provirus (11).

Retroviral IN proteins share distinctively conserved organization of their functional domain, and each contains a central catalytic core domain (CCD) flanked by N- and C-terminal domains (NTD and CTD) (12, 13). The NTD comprises the HHIC region (14) and mediates multimerization of IN and formation of a stable complex with viral DNA. The NTD is also necessary for recognizing and binding viral DNA (15, 16). Deletion mutants of this domain lose endonucleolytic and disintegration activities (17). The CCD harbors the most conserved region of INs and bears close structural homology to prokaryotic transposases (18). The enzyme active site is comprised of three invariant acidic residues (D, DX35E motif) (19). The CTD is the least conserved region and is able to bind DNA nonspecifically and is likely contained at the site for binding target DNA during integration (20, 21).

The biochemical characteristics of oncoretroviral and lentiviral integration reactions in vitro have been well reported and documented using synthetic duplex oligonucleotide substrates mimicking the U5 and U3 ends of retroviral DNA, whereas only a few studies on foamy viral IN have been reported (22, 23). We have characterized the biochemical properties of FFV IN using several metals containing the Mn2+ cation, which is a cofactor in the integration reaction (24). However, the FFV IN functional domains have not yet been clearly characterized. In this study, we constructed deletion and point mutants of FFV IN to analyze these functional domains. A complementation analysis indicated that the CCD of FFV IN has potential enzymatic activity but was unable to recognize viral or target DNA without the help of the NTD or CTD.
RESULTS

Construction, expression, and purification of IN mutants

FFV IN is larger than HIV-1 or PFV INs, with a molecular mass of 45 kDa and 383 amino acid residues. It has three domains called the CCD, NTD, and CTD. DNA constructs for preparing the point mutated INs were prepared by overlap extension polymerase chain reaction (PCR) (Fig. 1A).

Wild-type, deletion mutants, and point mutants were expressed in a vector containing the His-tag upstream of the multiple cloning site. The protein was purified using a Talon column and heparin or Ni-NTA column chromatography (Fig. 1B). Initially, we tried to purify FFV IN and its mutants using a Talon column, but some non-specific nuclease activity remained. So, we loaded the protein onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel, cut the desired band, and prepared a gel slice. The slice was mixed with SDS-running buffer prior to electro-elution. We purified the protein again by nickel-chelated chromatography with a small bed volume (0.25 ml) after dialyzing against the S10 buffer and collecting the supernatant. Finally, the FFV IN and mutant proteins were prepared from 2 L of an E. coli culture.

Complementation of FFV IN point mutants

Complementation reactions were performed to confirm whether the enzymatic activity of the CCD could be complemented with each point mutant. Seven kinds of point mutants were produced by overlap extension PCR (Fig. 1A), and D107A, D164H, and E200D were used in the complementation reaction. The combination of D107A and D164H showed all enzymatic activities. We speculate that the D164H point mutant had strong activity by itself. In contrast, the combination with E200D had no activity. These results suggest that if the second aspartic acid at 164 of the DDE motif in the CCD was defective, it can be supplied by adding a defective aspartic acid to 107 of the DDE motif, but that mutated glutamic acid at 200 strongly inhibited all enzymatic activities (data not shown).

Functional complementation of the defective deletion and FFV IN point mutants

The CCD is important for catalysis during IN activity. We combined the deletion mutants with other mutants and tested all possible combinations to investigate whether deletion mutants containing the CCD could increase enzymatic activity. The 3'-end processing reactions using the FFV U5 substrate are shown in Fig. 2A. Except the activity present in the NTD (N) and CCD (T), the other combinations did not increase activity. A similar pattern was observed for strand transfer activity (Fig. 2B). All combinations with CCD showed strong disintegration reaction activity. In particular, the combinations of N + TC, C + NT, and NT + TC showed strong activity. However, no 3'-end processing or disintegration activities were observed for the defective NTD, TC, or the combination of T + C (Fig. 2C). It seemed that the defective deletion mutants recover catalytic activity if combined with a different defective IN that compensates for its structural defects. Therefore, we suggest that the CCD of the FFV IN was not completely defective.

Several studies have shown that the DD(35)E mutants of retroviral INs lose enzymatic activities, as these residues are critical for activity. This was presumably the case for FFV IN, although this has not been tested directly. The lack of activity by the FFV IN deletion mutants may be due to an intact but non-functional CCD due to a lack of essential cooperation from the CTD with an appropriate conformation. We constructed seven FFV IN point mutants and five deletion mutants to investigate differences between the deletion and point mutants.

The point mutants D107A, D107H, and D164H alone had weak activity for 3'-end processing, whereas most of the other point mutants did not show any activity. Activities were inhibited or increased weakly when combined with the deletion mutants. However, some combinations such as NT + D107H increased activity (Fig. 2D), and, particularly, the complementation reactions between the NT and TC deletion mutants and the D107V point mutant increased activity significantly (Fig. 2E). All of the point mu-
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Fig. 2. In vitro complementation assays for 3'-end processing, strand transfer, and disintegration activities of the feline foamy virus (FFV) integrase deletion and point mutants. (A) 3'-end processing reactions of the deletion mutant integrases on an FFV U5 substrate. (B) Strand transfer activity of the deletion mutant integrases on an FFV U5 substrate. The strand transfer products (integration) appeared as bands larger than the 18mer. (C) Disintegration activities of the deletion mutant integrases on the FFV U5 substrate. (D, E) Complementation of 3'-end processing activities between deletion and point mutants. The 3'-end processing of D107A, D164H, E200A, E200D, and E200H data are not shown. (F) Complementation of disintegration activity between the deletion and D107A point mutant. (G) Complementation of disintegration activity between the deletion and D107H point mutants. (H) Complementation for disintegration activity between deletion and E200A point mutants. Disintegration data of D107V, D164H, E200D, and E200H are not shown.

In addition, we confirmed that the lack of the NTD resulted in no 3'-end processing or disintegration activities (except disintegration activity by TC). We also observed that different combinations of the CCD, NTD, or CTD had stronger disintegration activity compared to that of wild-type IN. This observation indicates that an intact FFV IN structure is not necessary for disintegration activity.

The point mutants lacking the CCD had no enzymatic activity, as the CCD, which is important for catalysis, was mutated. Loss of function of the DDE motif did not complement any of the point mutants. However, a mutated glutamic acid at 200 of the DDE motif resulted in inhibition of all enzymatic activities. This result indicates that the DDE motif glutamic acid at position 200 is more important for enzymatic activity than that of aspartic acid 107 or aspartic acid 164.

The complementation reactions using the point and deletion mutants also produced similar data as those reported previously. Almost all results showed a similar pattern with that of previous results, and the complementation reaction between D107V and the deletion mutants resulted in increased 3'-end processing activity. However, the E200A and E200H mutants did not show any enzymatic activities, as observed previously. But, E200A had disintegration activity when combined with the deletion mutants. This was probably due to structural changes by converting from a negatively charged amino acid to a nonpolar amino acid.

Several studies on FFV IN have been reported. However, the ac-
Table 1. Complementation of defective deletion and point mutants of FFV integrase

|        | D107A | | | D107H | | | D164H | | | E200A | | | E200D | | | E200H |
|--------|-------| | |       | | |       | | |       | | |       | | |       |
|        | 3’-end processing | Strand transfer | Disintegration | 3’-end processing | Strand transfer | Disintegration | 3’-end processing | Strand transfer | Disintegration | 3’-end processing | Strand transfer | Disintegration | 3’-end processing | Strand transfer | Disintegration |
| FFV IN | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + |
| T      | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| NT     | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| TC     | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |

|        | D107V | | | D164H | | | E200A | | | E200H |
|--------|-------| | |       | | |       | | |       |
|        | 3’-end processing | Strand transfer | Disintegration | 3’-end processing | Strand transfer | Disintegration | 3’-end processing | Strand transfer | Disintegration | 3’-end processing | Strand transfer | Disintegration |
| FFV IN | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + |
| T      | +/-  | -    | +    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| NT     | +    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| TC     | ++   | -    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |

|        | E200D | | | E200H |
|--------|-------| | |       |
|        | 3’-end processing | Strand transfer | Disintegration | 3’-end processing | Strand transfer | Disintegration |
| FFV IN | ++ + | ++ + | ++ + | ++ + | ++ + | ++ + |
| T      | -    | -    | -    | -    | -    | -    |
| NT     | -    | -    | -    | -    | -    | -    |
| TC     | -    | -    | -    | -    | -    | -    |

*The enzymatic activities of the wild-type integrase on its own DNA substrate were set at 100%, and -, +/-, +, ++, and +++ indicate 0-2, 2-5, 5-20, 20-70, and 70-100%, respectively of this activity.

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MATERIALS AND METHODS

Construction of the expression vector
The FFV IN expression vector was constructed by amplifying the FFV IN cDNA isolated from the FFV pol-gene and by ligating the PCR product into the BamHI and HindIII sites of the pQE9 expression vector. The resulting recombinant (pQE-FF) construct was characterized to contain six histidine codons upstream of the IN sequence. The presence of six histidines in the expressed protein provides for a simple purification method based on the selective affinity for cobalt-chelated adsorbent (24).

Cloning of the FFV IN deletion and point mutants
Five plasmids encoding deletion mutants were constructed by amplifying the IN coding region by PCR, digesting the amplified DNA with BamHI and HindIII, and ligating the cut DNA with BamHI-HindIII-digested pQE9 vector DNA. The oligonucleotides used to construct the mutants INs and the sequences of each primer are shown in Supplement Table 1. All PCRs were performed with pfu DNA polymerase (Beams Biotechnology, Chiang Mai, Thailand) to minimize DNA synthesis errors.

DNA fragments encoding point mutated INs were constructed using the overlap PCR method (28). The primer sequences are pro-
vided in Supplement Table 1. The first part of FFV IN containing the D107A mutation was amplified using FFVIn-1Val and FFVIn D107AA as primers and pQE-FF as the template. The second part of FFV IN containing the D107A mutation was amplified using FFVIn D107A and FFVIn-383SA as primers and pQE-FF as the template. The purified PCR products were mixed and used as a template in the second-round PCR to amplify the full-length D107A DNA, with FFVIn-1Val and FFVIn-383SA as primers. FFVIn-1Val and FFVIn D107HA and FFVIn D107H and FFVIn-383SA were used as primers for the first and second parts, respectively, to construct D107H. The procedures for D107V, D164H, E200A, E200D, and E200H used the same methods. The sequences of PCR-amplified DNA fragments were verified by restriction analysis and the diodeoxyxynucleotide chain termination method.

**Expression and purification of IN proteins**

The DNA constructs were transformed into *E. coli* XL-1 blue, and the transformants were grown at 37°C in 2 L of LB medium containing 50 μg ampicillin/ml. Isopropyl-l-thio-β-D-galactopyranoside (0.3 mM) was added at an optical density of 0.8 to induce expression, and the culture was grown for an additional 4 h. After harvesting, the cell pellet was frozen at −80°C, and the His-tagged proteins were purified by standard procedures and stored 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-me-captoethanol, 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 40,000 × g for 20 min at 4°C. The supernatant was directly loaded onto a Talen-column (bed volume of 1 ml, Clontech, Palo Alto, CA, USA) pre-equilibrated with S10 buffer (1 M NaCl and 10 mM CHAPS). The resin was washed four times with 3 ml of S10 buffer. Protein was eluted ten times with 0.5 ml S100 buffer (1 M NaCl and 100 mM CHAPS). Two methods were alternatively selected after purification. For the first method, fractions containing the protein were collected and diluted with nine volumes of buffer A [50 mM Tris··HCl (pH 7.6), 200 mM NaCl, 1 mM DTT, 0.1 mM EDTA, and 10% glycerol] before loading onto a column containing 0.25 ml heparin agarose. The column was washed with 6 ml of buffer A. The protein was eluted 12 times with 0.2 ml of buffer C [50 mM Tris··HCl (pH 7.6), 1 mM DTT, 0.1 mM EDTA, 1 M NaCl, 10% glycerol], and stored at −80°C. In the other method, the fractions were gathered and loaded on 12.5% or 16.5% SDS-polyacrylamide gels. Then, the gel containing the desired band was cut, sliced, and was added to SDS-running buffer (35 mM SDS, 1.5 M glycine, 250 mM Tris) prior to electro-elution using dialysis tube. The mixture was centrifuged at 2,500 × g for 10 min at 4°C. The supernatant was directly concentrated and dialyzed against S10 buffer using a concentrator (Thermo Scientific, Rockford, IL, USA). The remaining supernatant was collected and loaded onto a column of nickel-chelated nitri- triacetic acid agarose (bed volume of 0.25 ml, Clontech) pre-equi-

**In vitro FFV IN enzymatic activities**

The 3'-end processing, strand transfer, and disintegration activities of the deletion and point mutants were assayed as described previously (29). The following oligonucleotides were used as DNA substrates: FFVU5/20A, 5'-ACTGTCTGGCCCTATACCTG-3'; FFVU5/20S, 5'-CAGGGATAGGGCGACAGCT-3'; FFVU5/20S. The substrate was prepared by labeling the 5'-end of FFVU5/20S with [32P]ATP and T4 polynucleotide kinase and then annealing the labeled strands with their complementary oligonucleotides. Substrate for analyzing strand transfer was prepared by annealing the 5'-end labeled FFVU5/20S-2 with their complementary oligonucleotides. The FFV disintegration substrate (Y-oligomer) was prepared by annealing the 5'-end-labeled FT1 strand with the oligonucleotides FD4, FT2, and FFVU5/20A. The annealed Y-oligomer was purified on a 15% native polyacrylamide gel. In all assays, 0.1 pmol of DNA substrate was incubated with 3 pmol of IN for 90 min at 33°C in 10 μl of reaction buffer containing a final concentration of 20 mM HEPES (pH 7.5), 5 mM MnCl2, 30 mM NaCl, 10 mM DTT, 0.01 mM EDTA, and 0.05% Nonidet P40. One pmol of each of the two INs was premixed and added to 0.1 pmol of substrate for the complementation reactions. The reaction was stopped by adding 10 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF), and heated at 90°C for 3 min before electrophoresis on 15% polyacrylamide gels with 7 M urea in Tris-taurine EDTA buffer. The products were quantified with a Molecular Dynamic Phospholmage (Cyclone, PerkinElmer, Waltham, MA, USA).

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**REFERENCES**

1. Fauquet, C. M. and Fargette, D. (2005) International committee on taxonomy of viruses and the 3,142 unassigned species. *Virology*, 2, 64.
2. Saib, A. (2003) Non-primate foamy viruses. *Curr. Top. Microbiol. Immunol.* 277, 197-211.
3. Switzer, W. M., Salami, M., Shanmugam, V., Gao, F., Cong,
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M. E., Kuiken, C., Bhullar, V., Beer, B. E., Vallet, D., Gautier-Hion, A., Tooze, Z., Villinger, F., Holmes, E. C. and Heneine, W. (2005) Ancient co-speciation of simian foamy viruses and primates. Nature 434, 376-380.

4. Achong, B. G., Mansell, P. W., Epstein, M. A., and Clifford, P. (1971) An unusual virus in cultures from a human nasopharyngeal carcinoma. J. Natl. Cancer. Inst. 46, 299-307.

5. Geiselhart, V., Schwantes, A., Bastone, P., Frech, M. and Lochelt, M. (2003) Features of the Env leader protein and the N-terminal Gag domain of feline foamy virus important for virus morphogenesis. Virology 310, 235-244.

6. Mizuuchi, K. (1992) Polynucleotidyl transfer reactions in transpositional DNA recombination. J. Biol. Chem. 267, 21273-21276.

7. Brown, P. O., Bowerman, B., Varmus, H. E. and Bishop, J. M. (1989) Retroviral integration: structure of the initial covalent product and its precursor, and a role for the viral IN protein. Proc. Natl. Acad. Sci. U.S.A. 86, 2525-2529.

8. Fujiwara, T. and Mizuuchi, K. (1988) Retroviral DNA integration: structure of an integration intermediate. Cell 54, 497-504.

9. Engelman, A., Mizuuchi, K. and Craigie, R. (1991) HIV-1 DNA integration: mechanism of viral DNA cleavage and DNA strand transfer. Cell 67, 1211-1221.

10. Gerton, J. L., Herschlag, D. and Brown, P. O. (1999) Stereospecificity of reactions catalyzed by HIV-1 integrase. J. Biol. Chem. 274, 33480-33487.

11. Daniel, R., Katz, R. A. and Skalka, A. M. (1999) A role for DNA-PK in retroviral DNA integration. Science 284, 644-647.

12. Bushman, F. D., Engelman, A., Palmer, L., Wingfield, P. and Craigie, R. (1993) Domains of the integrase protein of human immunodeficiency virus type 1 responsible for polynucleotidyl transfer and zinc binding. Proc. Natl. Acad. Sci. U.S.A. 90, 3428-3432.

13. Engelman, A. and Craigie, R. (1992) Identification of conserved amino acid residues critical for human immunodeficiency virus type 1 integrase function in vitro. J. Virol. 66, 6361-6369.

14. Donehower, L. A. (1988) Analysis of mutant Moloney murine leukemia viruses containing linker insertion mutations in the 3' region of pol. J. Virol. 62, 3958-3964.

15. Ellison, V. and Brown, P. O. (1994) A stable complex between integrase and viral DNA mediates human immunodeficiency virus integration in vitro. Proc. Natl. Acad. Sci. U.S.A. 91, 7316-7320.

16. van den Ent, F. M., Vos, A. and Plasterk, R. H. (1999) Dissecting the role of the N-terminal domain of human immunodeficiency virus integrase by trans-complementation analysis. J. Virol. 73, 3176-3183.

17. Lutzke, R. A., Vink, C. and Plasterk, R. H. (1994) Characterization of the minimal DNA-binding domain of the HIV integrase protein. Nucleic Acids Res. 22, 4125-4131.

18. Dyda, F., Hickman, A. B., Jenkins, T. M., Engelman, A., Craigie, R. and Davies, D. R. (1994) Crystal structure of the catalytic domain of HIV-1 integrase: similarity to other polynucleotidyl transferases. Science 266, 1981-1986.

19. Goldgur, Y., Dyda, F., Hickman, A. B., Jenkins, T. M., Craigie, R. and Davies, D. R. (1998) Three new structures of the core domain of HIV-1 integrase: an active site that binds magnesium. Proc. Natl. Acad. Sci. U.S.A. 95, 9150-9154.

20. Engelman, A., Hickman, A. B. and Craigie, R. (1994) The core and carboxy-terminal domains of the integrase protein of human immunodeficiency virus type 1 each contribute to non-specific DNA binding. J. Virol. 68, 5911-5917.

21. Lodi, P. J., Ernst, J. A., Kuszekwii, J., Hickman, A. B., Engelman, A., Craigie, R., Clore, G. M. and Gronenborn, A. M. (1995) Solution structure of the DNA binding domain of HIV-1 integrase. Biochemistry 34, 9826-9833.

22. Oh, Y. T. and Shin, C. G. (1999) Comparison of enzymatic activities of the HIV-1 and HIV integrases to their U5 LTR substrates. Biochem. Mol. Biol. Int. 47, 621-629.

23. Pahl, A. and Flügel, R. M. (1993) Endonucleolytic cleavages and DNA-joining activities of the integration protein of human foamy virus. J. Virol. 67, 5426-5434.

24. Lee, D., Hyun, U., Kim, J. Y. and Shin, C. G. (2010) Characterization of biochemical properties of feline foamy virus integrase. J. Microbiol. Biotechnol. 20, 968-973.

25. Chow, S. A. and Brown, P. O. (1994) Substrate features important for recognition and catalysis by human immunodeficiency virus type 1 integrase identified by using novel DNA substrates. J. Virol. 68, 3896-3907.

26. Appa, R. S., Shin, C. G., Lee, P. and Chow, S. A. (2001) Role of the nonspecific DNA-binding region and alpha helices within the core domain of retroviral integrase in selecting target DNA sites for integration. J. Biol. Chem. 276, 45848-45855.

27. Pahl, A. and Flügel, R. M. (1995) Characterization of the human spuma retrovirus integrase by site-directed mutagenesis, by complementation analysis, and by swapping the zinc finger domain of HIV-1. J. Biol. Chem. 270, 2957-2966.

28. Horton, R. M., Ho, S. N., Pullen, J. K., Hunt, H. D., Cai, Z. and Pease, L. R. (1993) Gene splicing by overlap extension. Methods. Enzymol. 217, 270-279.

29. Shibagaki, Y. and Chow, S. A. (1997) Central core domain of the HIV-1 integrase is responsible for target site selection. J. Biol. Chem. 272, 8361-8369.