Molecular and Cellular Approaches to Regulation of Retroviral Infection for the Development of a New Gene Transfer Method

Yuuki OKINO

Contents

Chapter 1

General introduction

Introduction

Retroviral lifecycle

Since rouse sarcoma virus (RSV) was first discovered as a retrovirus in 1911 by F. P. Rous (1), a large number of retroviruses were isolated and there has been considerable research on the mechanisms of retroviral replication. Retroviruses have RNA genomes and their life cycles are arbitrarily divided into two distinct phases: the early and late steps (Fig. 1).

In the early step of retroviral infection, the viral enzyme reverse transcriptase (RT) produces double-stranded DNAs from the single-stranded RNA genomes. H. W. Temin and D. Baltimore discovered this enzyme in 1970 and this discovery made a big impact on the world since RT reaction went against the then-accepted central dogma. From the doma, transfer of genetic information is unidirectional: DNA → RNA → protein (1,2). Although limited DNA synthesis is occurred in viral particles before infection, this reaction usually occurs after the generation of the large nucleoprotein complex called reverse transcription complex (RTC) in the host cell cytoplasm. (3). During or shortly after the RT reaction, RTCs gradually transform into pre-integration complexes (PICs) (3). They contain the newly synthesized linear viral DNAs that are franked with long terminal repeats (LTRs). These viral DNAs are transported into host cell nucleus and integrated into the host cell chromosome by the viral enzyme integrrase (IN) (4).

Around 1990, the method for RTC/PIC purification was developed. Owing to this method, it was shown that not only viral proteins but also host cell proteins such as Ku80 (5), barrier-to-autointegration factor (6), lamina-associated polypeptide 2α (6), lens epithelium-derived growth factor/transcription co-activator p75 (LEDGF/p75) (7), high mobility group A (8), integrase interactor 1 (9) and many other proteins are determined as RTC/PIC components and these proteins are thought to have important roles in the early steps of retroviral replication.

After the integration, integrated viral DNAs are called proviruses and transcribed by the host cell RNA polymerase II. Transcribed viral RNAs are used for translation and progeny viral genomes. Viral proteins and RNAs assemble at the inner surface of the plasma membrane and immature viral particles bud from the surface of the cell. Shortly after budding, viral encoded protease cleaves viral polyproteins to produce the mature virions. It is thought that these late steps of retroviral replication are also affected by the PIC components. Daxx is a good example: Daxx is identified as the IN interacting protein that has no effects on the early steps of retroviral replication but repress the viral expression by recruiting histone deacetylase 1 (HDAC-1) after integration (10,11).

From this standpoint, identification of PIC components may be very important to understand the whole retroviral replication.

Retroviral vectors and gene therapy

While the understanding of retroviral replication was expanded, the generation of gene transfer system using retroviral particles became another active area of research. In

1983, the first successful gene transduction with retroviral vectors was reported (12). In this method, viral particles containing recombinant RNA genomes are produced and infected to target cells. Compared with other gene transfer systems, retroviral vectors have several advantages, including their ability to integrate transgene containing LTRs efficiently into host genome, to transduce variety of cell types, and to express the transgene at high levels. Now, retroviral vectors have become a standard tool for gene transfer technology.

In 1990, the first gene therapy for severe combined immunodeficiency caused by adenosine deaminase deficiency (ADA-SCID) using the retroviral vector was performed (13). This trial turned out to be successful and more than 1500 trials were performed since then. In these trials, however, some difficulties were found out.

First, these vectors are possible to disrupt the essential genes or promote the expression of oncogenes because the retroviral integration would occur in any position of the whole host genome. To solve this problem, some researchers tried to develop the retroviral vectors containing the fusion proteins consisting of IN and a sequence-specific DNA-binding protein in order to direct integration into a predetermined chromosomal region (14-18). However, these retroviral vectors failed to direct the retroviral integration efficiently because not only IN but also other PIC components would contribute to the integration site selection: LEDGF/p75 is the most informative regarding integration site selection (19-21). Thus, it is important to identify the PIC components and their functions to improve the efficiency of targeting the retroviral integration.

Second, integrated proviruses were often silenced especially in undifferentiated cells.

Daxx and HDAC-1 are thought to be the retroviral silencing factors (10,11). Although other cellular factors such as polycomb group protein EZH2 (22), histone lysine metyltransferase SUV39H1 (23,24) and G9a (25), DNA metyltransferase Dnmt1 (26), Dnmt3a and Dnmt3b (27), and TRIM family proteins TRIM28 (28) are also reported as retroviral silencing factors, detailed mechanisms have not been clarified. However, it is rational that PIC components are important roles in the retroviral silencing, as is the case of Daxx.

In this study, we searched for the moloney murine leukemia virus (MoMLV) IN-interacting cellular proteins to understand the mechanisms of integration site selection and retroviral silencing in more detail. By preliminary screening, we found that MoMLV IN interacted Yin Yang 1 (YY1), Promyelocytic leukemia (PML), and protein inhibitor of activated STAT (PIASy).

In Chapter 2, I focused on the interaction between retroviral INs and YY1. This protein was known to repress the HIV-1 and MoMLV expressions in the late step of infection by binding to LTR sequences (29-31). From the example of Daxx, we examined the hypothesis that YY1 had possibility to be a component of PIC and regulate the early steps of infection.

In Chapter 3, I focused on the interaction between MoMLV IN and PIASy or PML proteins. It is known that PIAS-interacting proteins are prone to modification by small ubiquitin-related modifier (SUMO) (32). Recently, it was reported that HIV-1 IN was SUMOylated and SUMOylation had important roles in the early step of infection (33). Thus, I hypothesize that MoMLV IN is also SUMOylated and PIASy catalyze this

reaction. SUMOylated proteins usually localized to the nuclear bodies organized by the PML proteins (34). Moreover, it is reported that PML proteins associate with HIV-1 PIC (9,35). However, PML function in the retroviral replication was controversial. Thus, I tried to clarify whether PML proteins regulate the retroviral replication and SUMOylation of IN affects their regulation.

References

1. **Javier, R. T., and Butel, J. S.** (2008) The history of tumor virology. *Cancer Res.* **68**, 7693-7706
2. **Baltimore D.** (1995) Discovery of the reverse transcriptase. *FASEB J.* **9**, 1660-1663
3. **Nisole, S., and Saïb, A.** (2004) Early steps of retrovirus replicative cycle. *Retrovirology* **1**, 9
4. **Suzuki, Y., and Craigie, R** (2007) The road to chromatin-nuclear entry of retroviruses. *Nat. Rev. Microbiol.* **5**, 187-196
5. **Li, L., Olvera, J. M., Yoder, K. E., Mitchell, R.S., Butler, S. L., Lieber, M., Martin, S. L., and Bushman, F. D.** (2001) Role of the non-homologous DNA end joining pathway in the early steps of retroviral infection. *EMBO. J.* **20**, 3272-3281
6. **Suzuki, Y., Yang, H., and Craigie, R.** (2004). LAP2α and BAF collaborate to organize the Moloney murine leukemia virus preintegration complex. *EMBO J.* **23**, 4670-4678
7. **Llano, M., Vanegas, M., Fregoso, O., Saenz, D., Chung, S., Peretz, M., and Poeschla, E. M.** (2004) LEDGF/p75 determines cellular trafficking of diverse lentiviral but not murine oncoretroviral integrase proteins and is a component of functional lentiviral preintegration complexes. *J. Virol.* **78**, 9524-9537
8. **Farnet, C. M., and Bushman, F. D.** (1997) HIV-1 cDNA integration: Requierment of HMG I (Y) protein for function of preintegration complexes. *J. Virol.* **65**, 1910-1915

9. **Turelli, P., Doucas, V., Craig, E., Mangeat, B., Klages, N., Evans, R., Kalpana, G., and Trono, D.** (2001) Cytoplasmic recruitment of INI1 and PML on incoming HIV preintegration complexes: interference with early steps of viral replication. *Mol. Cell* **7,** 1245-1254

10. **Huang, L., Xu, G. L., Zhang, J. Q., Tian, L., Xue, J. L., Chen, J.Z., Jia, W.** (2008) Daxx interacts with HIV-1 integrase and inhibits lentiviral gene expression. *Biochem Biophys Res Commun.* **373**, 241-245

11. **Geger, J. G., Katz, R. A., Ishov, A. M., Maul, G. G., Skalka, A. M.** (2005) The cellular protein Daxx interacts with avian sarcoma virus integrase and viral DNA to repress viral transcription. *J. Virol.* **79**, 4610-4618

12. **Onodera, M., Nelson, D. M., Sakiyama, Y., Candotti, F., and Blaese, R. M.** (1999) Gene therapy for severe combined immunodeficiency caused by adenosine deaminase deficieny: Improved retroviral vectors for clinical trials. *Acta Haematol.* **101**, 89-96

13. **Blaese, R. M., Culver, K. W., Miller, A. D., Carter, C. S., Fleisher, T., Clerici, M., Shearer, G., Chang, L., Chiang, Y., Tolstoshev, P., Greenblatt, J. J., Rosenberg, S.A., Klein, H., Berger, M., Mullen, C. A., Ramsey, W. J., Muul, L., Morgan, R. A., and Anderson, W. F.** (1995) T lymphocyte-directed gene therapy for ADA-SCID: Initial trial results after 4 years. *Science* **270**, 475-480

14. **Bushman, F. D.** (1994) Tethering human immunodeficiency virus 1 integrase to a DNA site directs integration to nearby sequences. *Proc. Natl. Acad. Sci. USA* **91**, 9233-9237

15. **Goulaouic, H., and Chow, S. A.** (1996) Directed integration of viral DNA

mediated by fusion proteins consisting of human immunodeficiency virus type 1 integrase and *Escherichia coli* LexA protein. *J. Virol.* **70**, 37-46

16. **Katz, R. A., Merkel, M., G. and Skalka, A. M.** (1996) Targeting of retroviral integrase by fusion to a heterologous DNA binding domain: in nitro activities and incorporation of a fusion protein into viral particles. *Virology* **217**, 178-190
17. **Bushman, F. D., and Miller, M. D.** (1997) Tethering human immunodeficiency virus type 1 preintegration complexes to target DNA promotes integration at nearby site. *J. Virol.* **71**, 458-464
18. **Tan, W., Dong, Z., Wilkinson, T. A., Barbas, C. F. 3rd, and Chow, S. A.** (2006) Human immunodeficiency virus type 1 incorporated with fusion proteins consisting of integrase and the designed polydactyl zinc finger protein E2C can bias integration of viral DNA into a predetermined chromosomal region in human cells. *J. Virol.* **80**, 1939-1948
19. **Ciuffi, A., Llano, M., Poeschla, E., Hoffman, C., and Leipzig, J.** (2005) A role for LEDGF/p75 in targeting HIV DNA integration. *Nat Med* **11**, 1287-1289
20. **Llano, M., Saenz, D. T., Meehan, A., Wongthida, P., and Peretz, M.** (2006) An essential role for LEDGF/p75 in HIV integration. *Science* **314**, 461-464
21. **Marshall, H. M., Ronen, K., Berry, C., Llano, M., and Sutherland, H.** (2007) Role of PSIP/LEDGF/p75 in lentiviral infectivity and integration targeting. *PLoS One* **2**, e1340
22. **Friedman, J., Chow, W.-K., Chu, C. K., Keedy. K. S., Archin, N.M., Margolis, D. M., and Karn, J.** (2011) Epigenetic silencing of HIV-1 by the histone H3 lysine 27 methltransferase enhancer of zeste 2. *J. Virol.* **85**, 9078-9089

23. **du Chene, I.** (2007) Suv39H1 and HP1gamma are responsible for chromatin-mediated HIV-1 transcriptional silencing and post-integration latency. *EMBO J.* **26**, 424-435

24. **Marban, C.** (2007) Recruitment of chromatin-modifying enzymes by CTIP2 promotes HIV-1 transcriptional silencing. *EMBO. J.* **26**, 412-423

25. **Imai, K., Togami H., and Okamoto, T.** (2010) Involvement of histone H3 lysine 9 (H3K9) metyltransferase G9a in the maintenance of HIV-1 latency and its reactivation by BIX01294. *J. Biol. Chem.* **285**, 16538-16545

26. **Walsh, C. P., Chaillet, J. R., and Bestor, T. H.** (1998) Transcription of IAP endogenous retrovirus is constrained by cytosine metylation. *Nat. Genet.* **20**, 116-117

27. **Okano, M., Xie, S., and Li, E.** (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) metyltransferases. *Net. Genet.* **19**, 219-220

28. **Wolf, D. and Goff, S. P.** (2007) TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. *Cell* **131**, 46-57

29. **Coull, J. J., Romerio, F., Sun, J.-M., Volker, J. L., Galvin, K. M., Davie J. R., Shi, Y., Hansen, U., and Margolis, D. M.** (2000) The human factors YY1 and LSF repress the human immunodeficiency virus type 1 long terminal repeat via recruitment of histone deacetylase 1. *J. Virol.* **74**, 6790-6799

30. **Flanagan, J. R., Becker, K. G., Ennist, D. L., Gleason, S. L., Driggers, P. H., Levi, B.-Z., Appella, E., and Ozato, K.** (1992) Cloning of a negative transcription factor that binds to the upstream conserved region of Moloney murine leukemia virus. *Mol. Cell. Biol.* **12**, 38-44

31. **He, G., and Margolis, D. M.** (2002) Counterregulation of chromatin deacetylation

and histone deacetylase occupancy at the integrated promoter of human immunodeficiency virus type 1 (HIV-1) by the HIV-1 repressor YY1 and HIV-1 activator Tat. *Mol. Cell. Biol.* **22**, 2965-2973

32. **Ryinki. M. M., Kaikkonen, S., Pehkonen, P., Jääskeläinen, T., and Palvimo J.J.** (2009) PIAS proteins: pleiotropic interactions associated with SUMO. *Cell. Mol. Life Sci.* **66**, 3029-3041

33. **Zamborlini, A., Coiffic, A., Beauclair, G., Delelis, O., Paris, J., Koh, Y., Magne, F., Giron, M. L., Tobaly-Tapiero, J., Deprez, E., Emiliani, S., Engelman, A., de Thé, H., and Saib, A.** (2011) Impairment of human immunodeficiency virus type-1 integrase SUMOylation correlates with an early replication defect. *J. Biol. Chem.* **286**, 21013-21022

34. **Lallemand-Breitenbach, V., and de Thé, H.** (2010) PML nuclear bodies. *Cold Spring Harb. Perspect. Biol.* 2, a000661

35. **Iordanskiy, S., Berro, R., Altieri, M., Kashanchi, F., and Bukrinsky, M.** (2006) Intracytoplasmic maturation of the human immunodeficiency virus type 1 reverse transcription complexes determines their capacity to integrate into chromatin. *Retrovirology* **3**, 4

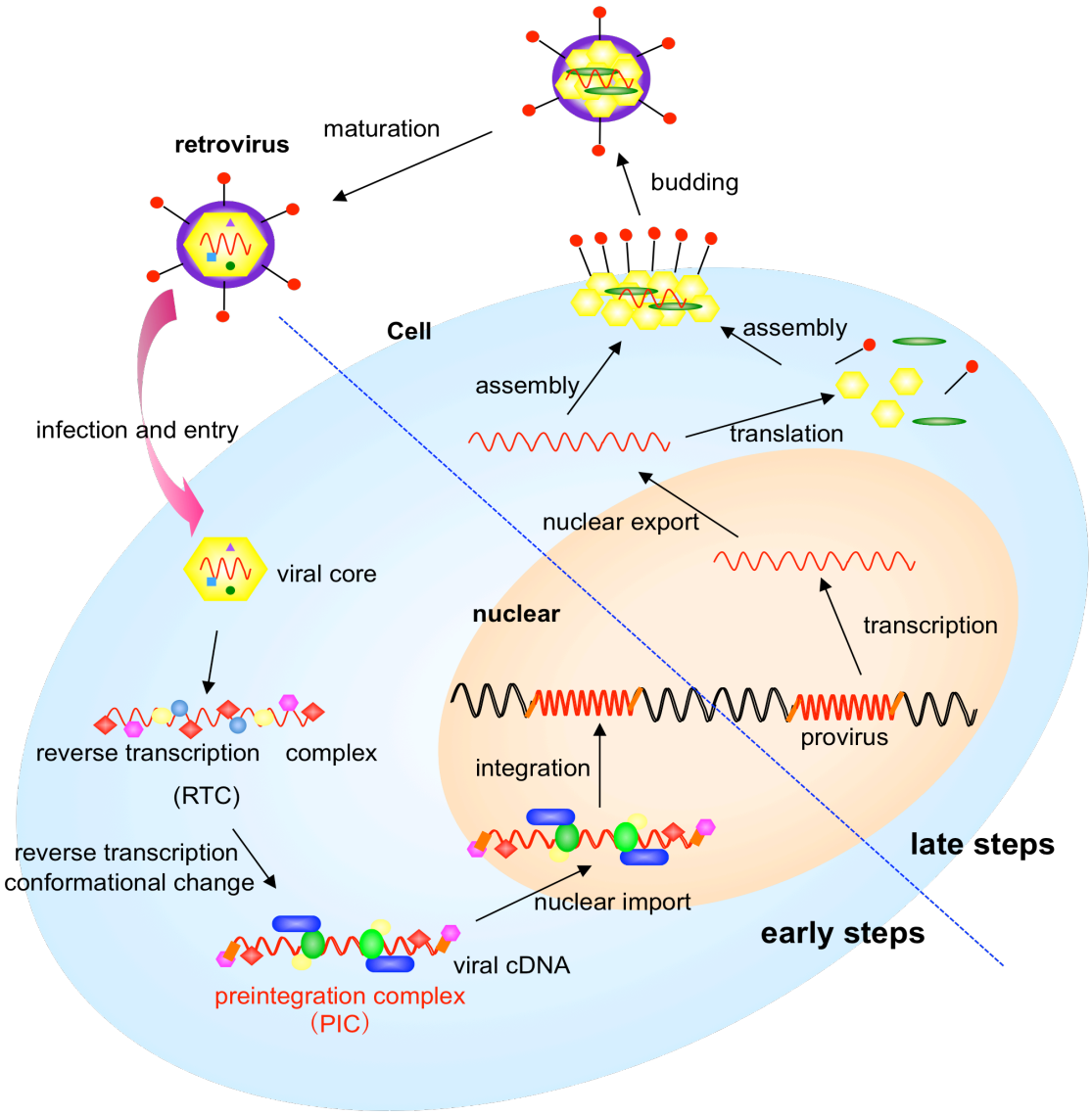

Figure. 1. **The retroviral life cycle**
A schematic view of early and late steps of the retroviral life cycle.

Chapter 2

Transcription factor YY1 interacts with retroviral integrases and facilitates moloney murine leukemia virus cDNA integration into the host chromosomes

Introduction

Following retroviral infection, reverse-transcribed viral cDNA is incorporated into preintegration complex (PIC) with viral proteins. Some examples include the human immunodeficiency virus type 1 (HIV-1) PIC with reverse transcriptase, integrase (IN), matrix, nucleocapsid and Vpr proteins, and the Moloney murine leukemia virus (MoMLV) PIC with reverse transcriptase, IN and capsid proteins. PIC is high molecular weight nucleoprotein complex (approximately 5,000 kDa) that is required for viral cDNA integration into the host genome (reviewed in reference 51). Depending on the virus type and cellular context, PICs or INs associate with several cellular proteins, such as barrier-to-autointegration factor (35, reviewed in reference 44), lamina-associated polypeptide 2α (52), lens epithelium-derived growth factor/transcription co-activator p75 (LEDGF/p75) (3, 5, 34), the SET complex (64), Ku80 (32), the human Polycomb group protein EED (embryonic ectoderm development gene product) (56) and Daxx (18). In addition, the HIV-1 virion contains integrase interactor 1 (INI1) (24, 69). Among these factors, a physical interaction has been reported between HIV-1 IN and INI1 (24, 69), EED (56) and LEDGF/p75 (3, 5, 34), as well as between avian sarcoma

virus (ASV) IN and Daxx (18). It has been recently reported that numerous cellular proteins including transcription factors, in addition to chromatin and RNA-binding proteins, potentially interact with MoMLV and HIV-1 INs (50).

Yin Yang 1 (YY1) is a sequence specific-DNA binding transcription factor that is an ortholog of Drosophila melanogaster Pleiohomeotic (Pho) and Pho like (reviewed in reference 53). YY1 is ubiquitously expressed in all tissues and highly conserved between Xenopus and human (reviewed in reference 17). It is able to activate and repress gene expression under different cellular contexts (reviewed in reference 46), as well as interacts with a wide variety of regulator proteins including retinoblastoma protein (40), histone acetyltransferase (p300/CBP) (28), histone deacetylase (HDAC1-3) (66-68), Sp1 (29), TATA-box binding protein (TBP), transcription factor (TF) IIB (1), YY1AP (57) and RYBP (16). In particular, YY1 can directly and indirectly bind to HIV-1 and MoMLV long terminal repeat (LTR) sequences, thereby repressing viral expression (7, 15, 19, reviewed in reference 22); for derepression of HIV-1 expression, HIV-1 Tat protein counteracts HDAC1 recruitment by YY1 and LSF to its LTR (19).

In this study, we demonstrated that YY1 is able to physically interact with MoMLV, HIV-1 and ASV INs and that it may associate with the MoMLV PIC. In vitro assays of IN activity and in vivo assessment of viral cDNA integration efficiency in YY1-knockdown cells revealed that YY1 facilitates the events required for viral cDNA integration into the host chromosomes.

Material and Methods

Vectors

Based on the NCBI database (accession number AF033811), the coding sequence of MoMLV IN (amino acids 2-408) was amplified from the gag-pol gene sequence in the pGP plasmid of the retrovirus packaging kit (Takara) by PCR with primers 5'-AATGGATCCGGAAAATTCATCACCCTACACC-3' and 5'-AATCTCGAGGGGGGCCTCGC-3', and then subcloned into pETBlue-2 (Novagen). ASV and HIV-1 INs were amplified by PCR from the pSRA2 (9) and pLP1 (Invitrogen) plasmids, respectively as templates, with primers for ASV: 5'-AAACCATGGCGCCCTTGAGAGAGGCTAAAGA-3' and 5'-AAACTCGAGTGCAAAAAGAGGGCTCG-3'; and for HIV-1: 5'-AAACCATGGCGTTTTTAGATGGAATAGATAAGGC-3' and 5'-AAACTCGAGATCCTCATCCTGTCTACTTGC-3'; the underlined sequences show the restriction enzyme recognition sites used for subcloning. The PCR fragments were subcloned into pETBlue-2.

For construction of the mammalian expression plasmid for MoMLV IN fused with the Flag epitope, MoMLV IN DNA from pETBlue2-MoMLV IN was amplified by PCR and ligated into the pFlag-CMV-2 plasmid (Sigma) at the *Eco*RI/*Bam*HI site. Deletion mutants of Flag-tagged MoMLV IN were also constructed by PCR, and the amplified DNA fragments were inserted into the pFlag-CMV-2 plasmid. For construction of expression plasmids for glutathione S-transferase (GST) fusion proteins of MoMLV, HIV-1 and ASV INs, PCR-amplified IN DNA fragments were inserted into pGEX-6P-2

or -5X-2 (GE Healthcare). The C209A MoMLV IN mutant (55) was generated by PCR-based site mutagenesis using pETBlue-2-MoMLV IN as the template with primers 5'-TGGAAATTACATGCTGCATACAGACCC-3' and 5'-GGGTCTGTATGCAGCATGTAATTTCCA-3' (the underlined sequences show the mutated nucleotides).

Human YY1 was amplified from cDNA of C33A cells, and its coding sequence was inserted into pGEX-6P-2 for expression as a GST fusion protein and into pcDNA4 (Invitrogen). His-tagged deletion mutants were generated from full-length human YY1 DNA by inserting the PCR-amplified fragments into pcDNA4/His C. The mutant DNA fragments were also subcloned into pGEX-6P-2.

Purification of GST-INs and the preparation of antibodies

Escherichia coli BL21 (DE3) containing the GST-MoMLV or -HIV-1 (amino acids 213-288) IN expression plasmid was grown in LB medium to an OD_{600} of 0.5 for MoMLV IN and an OD_{600} of 0.6 for HIV-1 IN. Then, 1 mM isopropyl-β-D-thiogalactoside was added, and *E. coli* was cultured at 25°C for 6 h for MoMLV and at 30°C for 6 h for HIV-1 INs. For the purification of MoMLV IN, the cells were suspended in phosphate buffered saline containing 1 μg/ml each of aprotinin, pepstatin and leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride, before being sonicated. For HIV-1 IN, the cells were harvested and suspended in phosphate buffered saline containing 1 mg/ml lysozyme. The cells were disrupted by sonication, and Triton X-100 was added at a final concentration of 1%. IN samples of MoMLV and HIV-1 were applied to a glutathione Sepharose 4B (GE Healthcare) column equilibrated with

phosphate buffered saline. After extensive washing with phosphate buffered saline, the GST fusion proteins were eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM glutathione. The anti-MoMLV and -HIV-1 IN antisera were raised against purified GST-IN preparations.

Immunoprecipitation

To determine the YY1-interacting regions of MoMLV IN, expression plasmids for Flag-MoMLV IN and deletion mutants were co-transfected with His-YY1 expression plasmid into 293FT cells using Lipofectamine 2000 (Invitrogen). Cells were then harvested between 36 and 48 h post-transfection, and lysed in buffer A (10 mM Tris-HCl [pH 7.4], 5 mM EDTA, 150 mM NaCl, 10% glycerol, 1% sodium deoxycholate and 1% Triton X-100) or buffer B (10 mM Tris-HCl [pH 7.4], 5 mM EDTA, 300 mM NaCl, 10% glycerol, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% sodium dodecyl sulfate (SDS)). After the cell lysate had been incubated with ANTI-FLAG M2 affinity gel (Sigma) for 4 h, the gel was washed twice with buffer A or B, and then an additional two times with Benzonase (a broad spectrum endonuclease from Serratia marcescens, Takara) buffer containing 20 mM Tris-HCl (pH 7.3), 50 mM KCl and 10 mM MgCl2. The gel was treated twice with 200 U/ml Benzonase, 100 U/ml DNase I and 5 μg/ml RNase A in Benzonase buffer at 37°C for 10 min and washed twice with buffer A or B. The samples were analyzed by western blotting using anti-His or -YY1 antibody. For identification of the IN-interacting regions in YY1, Flag-MoMLV IN and His-tagged derivatives of YY1 were expressed in 293FT cells followed by immunoprecipitation and western blotting. The anti-Flag and -His

antibodies were purchased from Sigma and Bethyl Laboratories, respectively. Anti-YY1 antibody (C-20) was obtained from Santa Cruz.

Preparation and analyses of PICs

To analyze whether YY1 associates with viral cDNA of MoMLV, NIH3T3 cells and MoMLV-producer clone #4 (a kind gift from Y. Suzuki, Kyoto University) (47) were co-cultured in DMEM containing 10% fetal calf serum for 16 h. NIH3T3 and producer cells were inoculated at a 4:1 ratio. PICs were prepared essentially as previously described (14). Briefly, infected cells were lysed with 20 mM HEPES-KOH (pH 7.5), 5 mM MgCl2, 150 mM KCl, and 1 mM dithiothreitol (DTT) containing 20 μg/ml aprotinin and 0.025% digitonin (Sigma), and the nuclei were removed by centrifugation. The cytoplasmic fraction was treated with 20 μg/ml of RNase A for 30 min at room temperature and subjected to gel filtration through a spin column of Sephacryl S-1000 (GE Healthcare). The partially purified PICs were incubated with anti-MoMLV IN antiserum, anti-YY1 antibody or control rabbit pre-immune serum for 4 h followed by absorption of the immunocomplex onto salmon sperm DNA-protein A agarose (Millipore) for 1 h. The beads were washed with buffer containing 20 mM HEPES-NaOH (pH 7.5), 5 mM MgCl2, 150 mM KCl, 6% Sucrose, 5 mM DTT, 6 mM EDTA and 0.1% NP-40. The resin was suspended in 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1% SDS and 200 μg/ml proteinase K and then incubated at 55°C for 1 h. Viral DNA was purified by phenol/chloroform extraction and isopropanol precipitation, and detected by Southern blotting using the LTR fragment of wild type

MoMLV (*ClaI*-*Nde*I fragment of the pNCA plasmid kindly provided by Y. Suzuki) as a probe.

Cytoplasmic fraction was also prepared from NIH3T3 cells infected with pQEGFP plasmid and its mutant at 4 h post-infection. After immunoprecipitation with anti-YY1 antibody, the eGFP sequence of the viral vector was detected by PCR with primers 5'-CGGCAACTACAAGACCCGC-3' and 5'-GAAGTTCACCTTGATGCCGTTC-3'.

In vitro binding assay

Various fragments of YY1, and MoMLV, HIV-1 and ASV INs were produced in *E. coli* BL21 or DH5α as GST fusions. A 1- to 2-μg aliquot of GST-MoMLV, -HIV-1, -ASV IN or their fragments was incubated with glutathione Sepharose 4B in binding buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 100 mM NaCl, 6% glycerol and 0.2% NP-40). The samples were treated with a mixture of Benzonase, DNase and RNase. His-tagged full-length YY1 treated with these nucleases was then mixed with each GST-IN fusion protein immobilized on the glutathione Sepharose beads in binding buffer and allowed to stand for 2 h at 4°C. The beads were washed three times with binding buffer and mixed with SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 5% 2-mercaptoethanol, 2% SDS, 5% sucrose and 0.002% bromophenol blue). The bound proteins underwent SDS-polyacrylamide gel electrophoresis (PAGE) followed by western blotting using anti-YY1 antibody. To determine the IN-interacting regions in YY1, similar pull-down experiments were performed with GST-YY1 fragments immobilized on the glutathione Sepharose 4B beads. Bound MoMLV IN was detected by western blotting using anti-MoMLV IN antiserum. His-tagged human YY1 was

purchased from Santa Cruz, and used for the in vitro integration and in vitro pull-down assays.

In vitro integration assay

His-tagged INs of MoMLV, its C209A mutant, ASV and HIV-1 were produced in *E. coli* BL21 (DE3) and purified using a Ni^{2+}-nitrilotriacetic acid agarose column as reported by Villanueva et al (55). Stocks of INs were diluted in 20 mM HEPES-KOH (pH 7.4), containing 100 mM KCl, 1.5 mM DTT and 10% glycerol for the assay. The following donor oligonucleotides were purchased from Hokkaido System Science: 5'-TTGACTACCCGTCAGCGGGGGTCTTTCA-3' (28 mer) and its complementary strand 5'-AATGAAAGACCCCCGCTGACGGGTAGTCAA-3' (30 mer) corresponding to the end of the U5 region of the MoMLV LTR; 5'-GTGTGGAAAATCTCTAGCA-3' (19 mer) and 5'-ACTGCTAGAGATTTTCCACAC-3' (21 mer) corresponding to the end of the U5 region of the HIV-1 LTR; and 5'-CTACAAGAGTATTGCATAAGACTACA-3' (26 mer) and 5'-AATGTAGTCTTATGCAATACTCTTGTAG-3' (28 mer) corresponding to the end of the U3 region of the ASV LTR. The shorter DNA fragments (100 pmol) were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Takara), purified on a column (Probe Quant G-50 Microcolumn, GE Healthcare), and annealed with an equivalent amount of the complementary strand. For the MoMLV IN assay, YY1, GST and IN (10 or 20 pmol) were preincubated in 20 μl IN dilution buffer at 4°C for 2 h before adding 2 pmol (1 μl) labeled substrate. It is possible that addition of proteins can non-specifically stimulate in vitro integration reactions. Thus, to eliminate the

non-specific effects, GST was added to ensure that the molar concentration of protein remained constant. After standing at 4°C for 30 min, 1.2 μg target DNA plasmid (1-2 μl; pBluescript II KS-, Stratagene) was added, and the reaction was started by the addition of concentrated buffer solution at a final concentration of 20 mM 2-(N-morpholino) ethanesulfonic acid (MES) (pH 6.2), 165 mM KCl, 10 mM MnCl2, 10 mM DTT and 10% dimethylsulfoxide (DMSO) (total volume: 35 μl). Molar ratios of preincubated IN:YY1 varied between 1:0.5 and 1:4. The same reaction buffer, but with a lower KCl concentration (120 mM), was used for the ASV IN assay, and the reaction buffer containing 20 mM HEPES-KOH (pH 7.2), 70 mM KCl, 10 mM MnCl2, 5 mM DTT, 10% DMSO and 10% PEG 6000 was used for the HIV-1 IN assay. For the enzymatic reaction of ASV and HIV-1 INs, various amounts of YY1, GST and IN (10 pmol) were preincubated in the reaction buffer at 4°C for 2 h. The ^{32}P-labeled substrates were then added, and the mixtures were allowed to stand for 30 min before the target DNA addition and enzymatic reaction. Reactions were performed at 37°C for 30 min. Reaction products were digested by protease (10 mM EDTA [pH 8.0], 0.5% SDS and 100 μg/ml proteinase K). After electrophoresis on a 1% agarose gel, integration products were detected by autoradiography.

The reaction product of MoMLV IN that showed mobility corresponding to the linear form of the target plasmid DNA was eluted from the gel, and the DNA was isolated. The DNA was amplified by PCR using KOD-Plus-Neo (Toyobo) and phosphorylated 28-mer donor DNA for the IN reaction as a primer. The enzyme in PCR buffer containing dNTPs was activated at 94°C for 2 min, and then the DNA sample was added. The mixture was stood at 68°C for 10 min in order to repair the gap

resulting from the integration reaction. After adding the primer, PCR was performed using the following protocol: 94°C for 15 sec, 55°C for 30 sec, and 68°C for 90 sec for a total 35 cycles. The resultant PCR product was ethanol precipitated, ligated using T4 DNA ligase, and then transformed into *E. coli* DH5α. The donor DNA for the IN reaction was designed so that it would form a *Mun*I site when the DNA fragments were inserted by two-end integration, and the target plasmid did not contain the restriction enzyme site. Thus, to confirm which clones had been produced by two-end integration, DNA prepared from each colony was cut with the enzyme. After rough estimation of the integration site by cutting the plasmid with *Ssp*I or *Pvu*II/*Eco*RI, the DNA sequence around the junction was determined. For analyses of the linear DNA product of the HIV-1 IN reaction, the following sequence was used for PCR: 5'-AAACCATGGTGTGGAAAATCTCTAGCA-3' (the underlined sequence shows the *Nco*I site). The target plasmid DNA did not contain an *Nco*I site.

Viral vectors

For the construction of the mouse stem cell virus (MSCV) vector pQEGFP, the eGFP DNA fragment was ligated into the *Eco*RI/*Cla*I site of pMSCVneo (Clontech), and the *Kpn*I fragment of the recombinant plasmid containing the viral sequence was isolated and ligated to pQCXIX (Clontech). For the construction of MoMLV-based viral vector pLNΔAG, the chicken actin promoter of the pMiwZ plasmid was ligated into the pLNRβ plasmid (25). The plasmid was then cut with *Not*I and ligated with the GFP fragment of pGREEN LANTERN-1 plasmid (Invitrogen). From this plasmid, the intron sequence in the actin promoter was deleted by PCR (25).

The YY1-binding site mutant of MSCV-derived viral vector pQEGFP was constructed by PCR with primers 5'-AGCTTAAGTAACGCACGTTTGCAAGGCATG-3' and 5'-CATGCCTTGCAAACGTGCGTTACTTAAGCT-3' (the underlined sequences show the mutated nucleotides).

The packaging cell line of viral vector pLNΔAG was established by transfecting pLNΔAG plasmid to GP293 cells followed by cloning. Viruses were produced by transfection of pVSV-G to packaging cells, or co-transfection of pQEGFP and pVSV-G to GP293 cells (21). Both the packaging and GP293 cells express the MoMLV gag-pol gene. Supernatants were collected 48 h post-transfection, and viruses were concentrated by centrifugation as previously described (21, 25, 38). GP293 and its derivative packaging cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% fetal calf serum, 1 mM sodium pyruvate and non-essential amino acids (Gibco). All virus preparations were passed through a 0.45-μm filter, and polybrene (8 μg/ml) was added for infection. Virus titer was measured using NIH3T3 cells as described previously (21, 25, 38).

Chromatin immunoprecipitation (ChIP) analysis

NIH3T3 cells infected with viral vector pLNΔAG were fixed with 1% formaldehyde at 4 h post-infection. ChIP assays using anti-YY1 antibody or -MoMLV antiserum were performed using a standard procedure with salmon sperm DNA-protein A agarose beads. DNA was fragmented by sonication to a mean size of 1 kb. The GFP sequence of pLNΔAG was detected by PCR with primers

5’-TTTTTCAAAGATGACGGGAACTACA-3’ and

5’-ATGCCATTCTTTTGCTTGTCGG-3’.

In vivo integration assay

NIH3T3 cells (4.0×10^4 cells) were seeded on 35-mm dishes and cultured for 24 h in DMEM containing 10% fetal calf serum. Mouse YY1 siRNA (#1006: 5’-GGCUGCACAAAGAUGUUCAGGGAUA-3’ and #1099: 5’-GCGUUCGUUGAGAGCUCAAAGCUAA-3’; 100 pmol each), or negative universal control siRNA (B-bridge), was introduced into the cells using Lipofectamine 2000. Cells were transfected again with siRNA 24 h after the first transfection. At 24 h after the second siRNA transfection, medium was changed to remove siRNA, and cells were infected with viral vector pQEGFP (MOI of 0.1) or pLNΔAG (MOI of 1.0). At 0, 4, 10, 24 and 48 h, cells were harvested for DNA extraction using the QIAamp DNA mini kit (Qiagen). The amount of total viral cDNA and two-LTR junction molecule (two-LTR circle) was measured by quantitative PCR (qPCR) with primers for two-LTR circle DNA: 5’-GCGCGCCAGTCCTCCGATAGAC-3’, and 5’-TACTTAAGCTAGCTTGCCAAACCTACAGG-3’ for pQEGFP; and 5’-GGAGGGTCTCCTCTGAGTGAT-3’ and 5’-CTCAGTTATGTATTTTTCCATGCCTT-3’ for pLNΔAG. For the detection of total viral cDNA for pLNΔAG and pQEGFP, primers were those used for the ChIP assay and immunoprecipitation of PICs, respectively. Genomic DNA was also purified 14 days post-infection using the Mag extractor Genome (Toyobo). The inserted viral DNA was

quantified by qPCR using the same primers as those used for total viral cDNA amplification.

The integrated viral DNA was also assessed by B1-nested PCR. For a first round of the qPCR, the primer for B1 sequence (5'-CTTTAATCCCAGCACTTGGGAGGC-3', the underlined C was biotinylated) and that for eGFP (5'-GAAGTTCACCTTGATGCCGTTC-3') or GFP (5'-ATGCCATTCTTTTGCTTGTCGG-3') were used. The PCR was performed under the following condition: 35 cycles of denaturation at 96°C for 15 s, annealing at 55°C for 30 s and extension at 68°C for 3 min. The amplified DNA samples were absorbed onto SA magnetic beads (Roche) and washed two times with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 1M NaCl, and two times with Tris-HCl (pH 7.5), 1 mM EDTA. After elution of the DNA from the beads with 0.1 M NaOH, sodium acetate was added for neutralization and the DNA was collected by ethanol precipitation. The recovered DNA was subjected to a second round of qPCR with primers for detection of the eGFP or GFP sequences.

Result

Physical interaction of INs with YY1

We searched for IN-interacting proteins, especially those involved in gene silencing, in order to elucidate the mechanism of retroviral silencing in undifferentiated cells. By preliminary screening involving immunoprecipitation of the extracts of cells co-expressing IN and silencing proteins, we found that MoMLV IN physically interacted with YY1. Thus, we expressed full-length MoMLV IN and its fragments as GST fusion proteins in *E. coli* (Fig. 1A) and then subjected them to a pull-down assay with His-YY1 to confirm their physical association. Analysis with anti-YY1 antibody demonstrated that full-length YY1 bound to the C-terminal and catalytic core domains of MoMLV IN (Fig. 1B); GST protein alone was used as a negative control. Comparison of band density to that of the input band showed that full-length IN tightly bound to YY1, while the C-terminal and catalytic core domains of MoMLV IN showed weaker interaction with YY1. Especially, the catalytic core domain bound to YY1 weakly. In these experiments, degradation was evident in all GST-IN preparations; however, the expression levels of intact fragments were similar for all IN derivatives.

To determine whether ASV and HIV-1 INs also physically interact with YY1, we next analyzed their GST-IN fusion proteins by pull-down assays. ASV IN could interact with YY1 (Fig. 1C), and based on the band density, the interaction appeared to be strong. For HIV-1 IN, its catalytic core and C-terminal domains bound weakly to YY1, but its N-terminal domain showed no interaction, as found for MoMLV IN. Obvious

interaction could not be detected between full-length IN and YY1 under this experimental condition. Although not fully clear, a form of steric hindrance may cause this weakened interaction between full-length IN and YY1. Immunoprecipitation of purified His-tagged full-length HIV-1 IN and His-YY1 demonstrated a weak interaction (Fig. 1D). These results for HIV-1 IN appeared to be in contrast to the MoMLV and ASV INs that showed tight binding between full-length INs and YY1. Taken together, the binding assay demonstrated that YY1 is able to physically interact with the three different INs, and thus the interactions might have a physiological significance. In these experiments, GST-INs, His-IN, and His-YY1 were extensively treated with Benzonase, and it was confirmed that a detectable level of nucleic acids were not contaminated by an agarose gel electrophoresis (data not shown). This may rule out the possibility that contaminating DNA or RNA bridged the two proteins and mediated the physical interaction (50).

To provide further evidence that the catalytic core and C-terminal domains of MoMLV IN interact with YY1, we also performed a detailed study on its YY1-binding regions by overexpressing YY1 and Flag-tagged MoMLV IN fragments (Fig. 1E) in 293FT cells, followed by immunoprecipitation using anti-Flag antibody and western blotting. As presented in Fig. 1F, YY1 bound to the C-terminal (290-408), but not to the N-terminal (2-116 and 2-135) fragments. Similar expression levels were found for these truncated IN constructs. IN fragments containing the whole or a part of catalytic core domain (112-289, 2-267, 2-154) also bound to YY1, but the band density was depending on fragments. The expression levels of these truncated INs also varied. Although fragment 155-267 contained a part of the catalytic core domain, the band

density was almost background level (Fig. 1F). These results are summarized in Fig. 1E. The immunocomplexes were treated with nucleases in all of these experiments. Although the interaction between the catalytic core domain of IN and YY1 seemed weak by pull-down assay, the results of immunoprecipitation demonstrated that a part of the catalytic core domain as well as the C-terminal domain of MoMLV IN interacted with YY1.

We next investigated which region of YY1 interacts with IN using in vitro pull-down assays. For these assays, we constructed several GST fusion proteins that were based on the domain structure of YY1 (Fig. 2A). YY1 comprises three domains including an N-terminal activation domain with a histidine-rich region, a central repressive domain with a glycine-rich region, and a C-terminal zinc-finger DNA-binding domain (reviewed in reference 53). As shown in Fig. 2B, YY1 fragments 1-224, 163-414 and 293-414, but not 1-142, bound to MoMLV IN, suggesting that it bound to the central repression and C-terminal domains of YY1. We also confirmed the location of the IN-binding regions with 293FT cells overexpressing Flag-MoMLV IN and truncated YY1 fragments. Figure 2C shows that YY1 fragments 1-224, 163-414 and 225-414 were co-immunoprecipitated with IN, but fragment 1-142 was not precipitated. Since bands of the fragments 163-414 and 225-414 were weak in intensity (Fig. 2C, center panel), immunoprecipitation was carried out under different condition (high stringency) with IN minus controls (Fig. 2C, right panel). Fragments (163-414 and 225-414) bound to IN. Pull-down and immunoprecipitation experiments consistently showed that the central repressive domain of YY1, possibly including the GA and GK regions, bound to IN, while the results for C-terminal DNA-binding domain were

slightly different. That is, the interaction appeared weak by immunoprecipitation compared to pull-down assays. The reason for this slight discrepancy remains unclear. However, binding of the other protein factors or post-translational modifications may have hampered the physical interaction between the DNA-binding domain and IN in the immunoprecipitation experiments, as protein factors such as p300/CBP (28) and HDAC (66-68) bind to this domain, and the amino acids residues 261-333 in addition to 170-200 contain acetylation sites (68). Another possible cause of the discrepancy is differences in the stringency of the binding assays.

In vivo association of YY1 with MoMLV cDNA

As YY1 can potentially form physical interactions with three different INs, we next examined whether YY1 associates with viral DNA in the cytoplasm of infected cells. The cytoplasmic fraction of NIH3T3 cells infected with wild type MoMLV was subjected to gel filtration and immunoprecipitation, and viral sequence was detected by Southern blotting using a viral cDNA fragment as a probe. As presented in Fig. 3A, a viral sequence of the expected size was detected in the precipitates of both anti-YY1 antibody and anti-MoMLV IN antiserum, demonstrating that YY1 as well as IN associated with viral cDNA in the cytoplasm of the infected cells. As judged by qPCR, 0.35% (YY1) and 1% (IN) of the input DNA was recovered by immunoprecipitation.

We then also examined the association of YY1 with viral DNA in NIH3T3 cells that had been infected with a replication defective viral vector. At 4 h post-infection, the cells were collected and the association between YY1 and viral DNA was studied by

ChIP; anti-MoMLV IN antiserum was used as a positive control. As shown in Fig. 3B, viral DNA could be detected both with anti-YY1 antibody and anti-MoMLV IN antiserum.

It has been reported previously that both MoMLV (15) and HIV-1 (7) contain YY1-binding motifs in their LTR sequences, suggesting that YY1 may be recruited specifically to this sequence and associated with the PIC. To verify that the association of YY1 with viral DNA is independent of the LTR, a mutated viral vector based on MSCV (pQEGFP) that contains a mutation in the YY1-binding site of the LTR sequence was constructed as reported (Fig. 3C) (reviewed in reference 22). The LTR sequence of non-mutated pQEGFP differed from that of MoMLV by two nucleotides; however, these changes were found outside the YY1-binding site. NIH3T3 cells were infected with the VSV-G-pseudotyped viral vector with or without this mutation. At 4 h post-infection, the cytoplasmic fractions were immunoprecipitated with anti-YY1 antibody, and the viral DNA in the precipitate was amplified by PCR. As indicated in Fig. 3D, similar levels of viral DNA could be detected for both wild type and mutated viruses by PCR. This finding demonstrated that the association of YY1 with viral DNA was mainly mediated by physical interaction with MoMLV IN and not through binding to the YY1-binding sequence in the LTR. As cellular distribution of YY1 is cell cycle dependent and YY1 localizes to the cytoplasm during the G1 and early S phase (39), it seems reasonable that cytoplasmic YY1 associates with viral DNA.

YY1 activates in vitro integration

We next investigated whether the physical interaction between YY1 and INs modulates retroviral integration. We first analyzed the in vitro IN activity in both the presence and absence of YY1 using His-tagged MoMLV, HIV-1 and ASV INs produced in *E. coli*. The MoMLV IN activity (strand transfer activity) was measured using a short LTR sequence that was labeled with ^{32}P at the 5' end as the donor and plasmid pBluescript II KS^{-} as the target. IN and YY1 were pre-mixed at 4°C for 2 h followed by incubation with donor DNA at 4°C for 30 min, and the reaction was initiated upon addition of target DNA and concentrated reaction buffer (protocol 1 in Fig. 4A). Under the assay condition, two forms of integration products were observed. Band 1 in Fig. 4A, the mobility of which on agarose gel electrophoresis was similar to that of the open circular target plasmid, was supposed to be donor-tagged open circular target DNA, e.g., a one-end integration product. The mobility of Band 2 corresponded to that of the linear form of the target DNA. From this band, the DNA was isolated, PCR amplified, self-ligated, and introduced into *E.coli*. After confirming that the cloned DNA was resulted from the two-end integration by *Mun*I digestion, the sequences of the DNA were determined. Sequence analysis of the donor-target junctions of the DNA revealed that around 70 to 75% of products contained the correct 4-bp duplication, irrespective of the presence of YY1, as shown in Table 1. From a comparison of the amounts of integration products, we found that YY1 activated integration (Fig. 4A). Four times excess amounts of YY1 did not result in any further activation and partially impaired enzyme activity (data not shown). To confirm this activation, IN activity was also assessed using a different pre-incubation protocol (protocol 2), which resulted in similar activation (Fig. 4B). This finding suggested that YY1 may not exhibit

significant effects on IN-donor DNA interaction. Furthermore, activation of IN mutant C209A that shows a higher oligomerization state (equilibrium between the dimer and tetramer) and has an increased solubility (55), was also observed (data not shown). Taken together, these results indicated that MoMLV IN can be activated by a relatively low concentration of YY1.

With HIV-1 IN, a band with mobility that corresponded to the linear form of the target DNA (Band 2 in Fig. 4C) appeared in the presence of YY1. The mobility of Band 1 matched that of donor-tagged open circular target plasmid DNA. From Band 2, DNA was isolated, PCR amplified, and introduced into *E. coli*. The cloned DNA from almost all colonies did not contain a two-end concerted integration product. This result agrees with the previous observation that two-end concerted integration occurred at very low frequency when short donor fragments were used (33). Activation was also observed for HIV-1 IN, in which YY1 activated the IN reaction by more than 10-fold at an IN:YY1 ratio of 1:2 when compared the density of Band 1 (Fig. 4C). GST protein slightly activated IN reaction (<1.5-fold, lane 6). This result suggests that relatively weak interaction between HIV-1 IN and YY1 substantially affected IN activity (Fig. 1C and D). In contrast to the results for MoMLV, excess amounts of YY1 versus IN (approximately 8 times) activated HIV-1 IN in a dose-dependent manner, possibly due to the weak interaction between the two molecules (data not shown). In addition to HIV-1 IN, ASV IN showed 4-5 fold activation in the presence of YY1 judging from the density of the band corresponding to donor-tagged open circular target DNA (data not shown). The purity of the IN preparations in these experiments was high, although a

small amount of degradation was observed (Fig. 4A and C). These results demonstrated that the three INs are activated by YY1, most likely through their physical interactions.

In vivo integration assay

To investigate the physiological importance of the IN-YY1 interaction, we assessed whether YY1 affects in vivo integration events using siRNA-mediated knockdown of YY1. VSV-G-pseudotyped MoMLV and MSCV viral vectors were prepared using cell lines that express wild type MoMLV *gag-pol*. YY1-knockdown or control siRNA-transfected NIH3T3 cells were infected with these viral vectors for analysis of total viral cDNA, two-LTR circles and the integrated form of viral DNA. Following knockdown of YY1, the amount of YY1 in NIH3T3 cells was reduced to less than 20% of that in control cells (Fig. 5A). The amount of viral cDNA peaked at approximately 10 h post-infection for both viral vectors, irrespective of the presence of siRNA, and then gradually decreased (Fig. 5B). The total amount of synthesized viral cDNA in knockdown cells with both viral vectors was increased 1.8- to 2-fold that in control cells at 10 h, suggesting that reverse transcription was partly abrogated by YY1. Previously, it was shown that the C-terminal region of HIV-1 IN binds to reverse transcriptase, and mutations in the C-terminal region of HIV-1 and MoMLV INs hamper the reverse transcriptase activity (8, 27, 58, 62). Since YY1 binds to the C-terminal region of the MoMLV IN, it is possible that YY1 may affect reverse transcriptase activity. Alternatively, we cannot rule out the possibility that YY1 directly affects the enzymatic activity. In this regard, it is noteworthy that YY1 binds to single- and double-stranded

RNA with a certain sequence preference for the oligo U-stretch of single stranded and A:U-stretch of double stranded RNA based on the analysis of messenger ribonucleoprotein particle synthesis (2). Thus, it is possible that YY1 may be able to bind to viral RNA and hamper reverse transcription. So far, we have not yet obtained any clear result that elucidates the mechanism of the reduction of cDNA synthesis by YY1. Further extensive studies are necessary to clarify the mechanism and physiological meaning of this effect of YY1.

We next examined the effects of YY1 on the two-LTR circle, which is thought to be the result from direct end-to-end joining of the viral DNA and is dead end product (45). For PCR, primers annealing to the U5 and U3 regions of the LTR of pLNΔAG or the R and U3 regions of pQEGFP were used. The levels of two-LTR circle increased by 24 h, after which they either gradually increased or decreased depending on the viral vector used (Fig. 5C). The mean copy number of the two-LTR circle was less than 1% of total viral cDNA at 24 h post-infection in both knockdown and control cells. siRNA facilitated the two-LTR circle formation by 1.3- to 2.9-fold compared to control cells at 24 h. Two-LTR circle formation has been reported to prevent apoptosis in infected cells that is induced by non-integrated linear viral DNA, and this formation can be used as an index for nuclear transport of PICs (31, 42). However, whether two-LTR circle formation serves as a true reflection of nuclear entry of PICs remains controversial, as two-LTR circle was detected in the cytoplasm of MLV-infected aphidicolin-arrested NIH3T3 cells (45). Although the physiological mechanisms for this reduction in two-LTR circle formation by YY1 remain to be elucidated, the effects of YY1 may be secondary to the reduction in viral cDNA synthesis.

As presented in Fig. 5D, the amount of viral sequence measured by qPCR 14 days after infection which probably corresponded to the amount of integrated form of viral DNA, decreased 3- to 6-fold by knockdown of YY1 with both the MoMLV- and MSCV-based vectors, suggesting that YY1 facilitates in vivo integration events. Similar results were obtained by nested PCR 24 h post-infection (Fig. 5E). This result is in contrast to the viral cDNA synthesis, which increased following siRNA treatment (Fig. 5B). As the nuclear entry of the MoMLV PIC is dependent on the disappearance of the nuclear envelope during cell growth (31, 42), and is required for successive integration events, growth retardation following siRNA treatment was evaluated. We found that cell growth was not significantly affected by siRNA as assessed by direct counting of cell numbers in parallel runs. We observed that the increase in cell number in the siRNA-treated dishes was 60 to 80% of that observed in the control culture during the first 24 h after virus infection. Afterward, obvious retardation of cell growth was not observed (data not shown). We hypothesized that this level of growth retardation did not profoundly influence nuclear entry of PIC and its subsequent integration, although we could not rule out a limited effects on integration. We also studied the expression of virally encoded GFP. As demonstrated in Fig. 5F, the mean intensity of GFP fluorescence in the knockdown cells was increased 1.4-fold at 48 h post-infection, although no increase was observed at 24 h. This suggests that the expression of GFP was partly repressed by YY1. Taken together, siRNA appears to facilitate viral cDNA synthesis, while abrogating integration. Thus, we speculated that YY1 activates integration events in vivo, even though the reverse transcriptase reaction was partly inhibited.

Discussion

The present study demonstrated that YY1 is able to bind to MoMLV, HIV-1 and ASV INs. This interaction was found to occur at the catalytic core and C-terminal domains of the MoMLV and HIV-1 INs. IN proteins can be divided into three functional domains. The N-terminal domain contains a conserved His-His/Cys-Cys (HHCC) that is involved in protein multimerization (65, 70); the catalytic core domain contains a conserved DDE motif that is essential for catalytic activity (11, 12, 26); and the less conserved C-terminal domain is involved in non-specific DNA binding activity (13, 59). The amino acid sequence of MoMLV IN has extra sequences at both the C- and N-termini, and an additional 36 amino acid sequence in the C-terminal region that is close to the boundary of the catalytic core domain. In addition, various amino acids differ in the common region from those of HIV-1 and ASV INs (23, 41). Despite the diversity in their amino acid sequence, MoMLV and HIV-1 INs share a similar array of helix and b-strand structures (41), suggesting that interaction with YY1 may rely on their secondary rather than primary structure.

YY1 could not be detected in MoMLV virions although IN was detected (Mizutani: unpublished result). The immunoprecipitation experiments shown in Fig. 3 demonstrated that YY1 associates with viral cDNA in the cytoplasm of infected cells. These results suggest that YY1 may associate with PIC in the cytoplasm. Since viral cDNA synthesis was partly hampered by YY1, it is possible that YY1 may interact with IN before PIC formation. Extensive analysis is necessary to clarify this.

We also found that YY1 stimulated in vitro integration reactions of MoMLV, HIV-1 and ASV INs. Maximum activation was observed for the IN/YY1 molar ratio of 1:2 with MoMLV and a higher ratio with HIV-1 IN. Thus, an intriguing question is how YY1 facilitates integration reaction in vitro. As YY1 showed an apparent positive effect on the in vitro activity of three different INs, we assumed that YY1 might have a common and fundamental function for basic IN catalytic mechanisms. In the present study, it was demonstrated that the distribution of host target duplication of two-end concerted integration was not affected by YY1. And the MoMLV IN C209A mutant, which shows a higher oligomerization state, was also activated by YY1, suggesting that the activation may not result from the changes in oligomerization state of IN. However, we have not yet obtained any direct evidence to demonstrate the molecular basis of this activation. In this relationship, it is interesting that YY1, together with the INO80 chromatin-remodeling complex, is essential for mammalian homologous recombination and was shown to bind to a recombination intermediate termed the holiday junction in vitro (60). Based on the results of this study, an attractive hypothesis would be that YY1 might facilitate catalytic reactions by stabilizing specific reaction intermediates. Further analyses on this point will provide us with a better understanding of the mechanisms underlying the enzymatic reaction of IN. We also demonstrated that YY1 facilitated the IN-mediated integration events in vivo, even though viral cDNA synthesis was impaired. This positive effect on in vivo integration may in part mirror the activation of in vitro IN activity by YY1.

Retroviruses display preferences for target DNA sequences in the immediate vicinity of the integration site, which appear to be virus specific, and IN may select

specific sequences (10, 20). In addition to this nucleotide-scale selection of target sites, previous studies have demonstrated that viruses have favored and disfavored chromosomal regions for integration. For instance, chromosomal regions rich in expressed genes are favored for HIV-1 integration (10, 30, 37, 48). MoMLV prefers integration near the start sites of transcription, and CpG islands that are genomic regions enriched in the rare CpG dinucleotide and commonly associated with the TATA box-less transcription start site (10, 37, 54, 61). IN is supposed to be the principal viral determinant of the integration specificity probably through tethering to cellular proteins bound near preferred genomic regions (30). LEDGF/p75 is considered to act as a tethering factor for HIV-1 PIC (6, 36, 48). YY1-binding sites have been shown to reside in promoter and translation start sites with certain frequency (above 3% of human promoters contain the binding site) (63). Moreover, YY1 interacts with basal transcription machinery such as TBP and TFIIB (1), and forms physical complexes, suggesting that YY1 may modulate the access of MoMLV PIC to chromosomes. This might cause bias for integration site selection to a certain extent. However, there is no clear evidence that MoMLV integration sites are enriched in close vicinity of YY1-binding sites by now.

In *Drosophila*, the gene known as *Pho*, an ortholog of YY1 binds to the *Polycomb* responsive elements of the *Hox* and *Engrailed* genes, and recruits the *Polycomb* repressive complex to the genes. A similar function in mammals has not been reported for YY1 (reviewed in reference 49). Instead, YY1 was found to interact with the *Polycomb* protein *Ezh2* that shows histone lysine methyltransferase activity (4) and is a member of *Polycomb* repressive complex 2 with EED. In *Xenopus*, YY1 interacts with

EED (43), and EED physically interacts with HIV-1 IN (56). Thus, it is possible that these repressive proteins may interact with PIC, as in the case for HDAC (19), and may be eventually delivered to integration sites and repress the integrated proviral DNA under some physiological conditions. In the present case, YY1 seemed to partially repress GFP expression, but the molecular basis of its action and its relationship to other repressive proteins remains unclear.

Although no direct evidence for the physiological functions of the IN-YY1 interaction has been found except for stimulation of in vitro and in vivo integration reaction, we hypothesized that YY1 plays an important role in integration and related events that occur after integration. Detailed analyses are required to clarify the precise biological function of the IN-YY1 interaction.

Acknowledgements

This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) by Bio-oriented Technology Research Advancement Institution (BRAIN).

References

1. **Austen, M., B. Lüscher, and J. M. Lüscher-Firzlaff.** 1997. Characterization of the transcriptional regulator YY1. J. Biol. Chem. **272:**1709-1717.
2. **Belak, Z. R., and N. Ovsenek.** 2007. Assembly of the Yin Yang 1 transcription factor into messenger ribonucleoprotein particles requires direct RNA binding activity. J. Biol. Chem. **282:**37913-37920.
3. **Busschots, K., A. Voet, M. De Maeyer, J.-C. Rain, S. Emiliani, R. Benarous, L. Desender, Z. Debyser, and F. Christ.** 2007. Identification of the LEDGF/p75 binding site in HIV-1 integrase. J. Mol. Biol. **365:**1480-1492.
4. **Caretti, G., M. Di Padova, B. Micales, G. E. Lyons, and V. Sartorelli.** 2004. The Polycomb Ezh2 methyltransferase regulates muscle gene expression and skeletal muscle differentiation. Genes Dev. **18:**2627-2638.
5. **Cherepanov, P., G. Maertens, P. Proost, B. Devreese, J. Van Beeumen, Y. Engelborghs, E. De Clercq, and Z. Debyser.** 2003. HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells. J. Biol. Chem. **278:**372-381.
6. **Ciuffi, A., M. Llano, E. Poeschla, C. Hoffmann, J. Leipzig, P. Shinn, J. R. Ecker, and F. Bushman.** 2005. A role for LEDGF/p75 in targeting HIV DNA integration. Nat. Med. **11:**1287-1289.
7. **Coull, J. J., F. Romerio, J.-M. Sun, J. L. Volker, K. M. Galvin, J. R. Davie, Y. Shi, U. Hansen, and D. M. Margolis.** 2000. The human factors YY1 and LSF

repress the human immunodeficiency virus type 1 long terminal repeat via recruitment of histone deacetylase 1. J. Virol. **74:**6790-6799.

8. **Dar**, **M. J., B. Monel**, **L. Krishnan**, **M.-C. Shun**, **F. Di Nunzio**, **D. E. Helland, and A. Engelman.** 2009. Biochemical and virological analysis of the 18-residue C-terminal tail of HIV-1 integrase. Retrovirology. **6:** 94.
9. **DeLorbe, W. J., P. A. Luciw, H. M. Goodman, H. E. Varmus, and J. M. Bishop.** 1980. Molecular cloning and characterization of avian sarcoma virus circular DNA molecules. J. Virol. **36:**50-61.
10. **Derse, D., B. Crise, Y. Li, G. Princler, N. Lum, C. Stewart, C. F. McGrath, S. H. Hughes, D. J. Munroe, and X. Wu.** 2007. Human T-cell leukemia virus Type 1 integration target sites in the human genome: comparison with those of other retroviruses. J. Virol. **81:**6731-6741.
11. **Drelich, M., R. Wilhelm, and J. Mous.** 1992. Identification of amino acid residues critical for endonuclease and integration activities of HIV-1 IN protein in vitro. Virology **188:**459-468.
12. **Engelman, A., and R. Craigie.** 1992. Identification of conserved amino acid residues critical for human immunodeficiency virus type 1 integrase function in vitro. J. Virol. **66:**6361-6369.
13. **Engelman, A., A. B. Hickman, and R. Craigie.** 1994. The core and carboxyl-terminal domains of the integrase protein of human immunodeficiency virus type 1 each contribute to nonspecific DNA binding. J. Virol. **68:**5911-5917.
14. **Engelman, A., I. Oztop, N. Vandegraaff, and N. K. Raghavendra.** 2009. Quantitative analysis of HIV-1 preintegration complexes. Methods **47**:283-290.

15. **Flanagan, J. R., K. G. Becker, D. L. Ennist, S. L. Gleason, P. H. Driggers, B.-Z. Levi, E. Appella, and K. Ozato.** 1992. Cloning of a negative transcription factor that binds to the upstream conserved region of Moloney murine leukemia virus. Mol. Cell. Biol. **12:**38-44.

16. **García, E., C. Marcos-Gutiérrez, M. del Mar Lorente, J. C. Moreno, and M. Vidal.** 1999. RYBP, a new repressor protein that interacts with components of the mammalian Polycomb complex, and with the transcription factor YY1. EMBO J. **18:**3404-3418.

17. **Gordon, S., G. Akopyan, H. Garban, and B. Bonavida.** 2006. Transcription factor YY1: structure, function, and therapeutic implications in cancer biology. Oncogene **25:**1125-1142.

18. **Greger, J. G., R. A. Katz, A. M. Ishov, G. G. Maul, and A. M. Skalka.** 2005. The cellular protein Daxx interacts with avian sarcoma virus integrase and viral DNA to repress viral transcription. J. Virol. **79:**4610-4618.

19. **He, G., and D. M. Margolis.** 2002. Counterregulation of chromatin deacetylation and histone deacetylase occupancy at the integrated promoter of human immunodeficiency virus type 1 (HIV-1) by the HIV-1 repressor YY1 and HIV-1 activator Tat. Mol. Cell. Biol. **22:**2965-2973.

20. **Holman, A. G., and J. M. Coffin.** 2005. Symmetrical base preferences surrounding HIV-1, avian sarcoma/leukosis virus, and murine leukemia virus integration sites. 2005. Proc. Natl. Acad. Sci. USA **102**: 6103-6107.

21. **Hotta, A., Y. Saito, K. Kyogoku, Y. Kawabe, K. Nishijima, M. Kamihira, and S. Iijima.** 2006. Characterization of transient expression system for retroviral vector production. J. Biosci. Bioeng. **101:**361-368.

22. **Iba, H., T. Mizutani, and T. Ito.** 2003. SWI/SNF chromatin remodelling complex and retroviral gene silencing. Rev. Med. Virol. **13:**99-110.

23. **Johnson, M. S., M. A. McClure, D.-F. Feng, J. Gray, and R. F. Doolittle.** 1986. Computer analysis of retroviral *pol* genes: assignment of enzymatic functions to specific sequences and homologies with nonviral enzymes. Proc. Natl. Acad. Sci. USA **83:**7648-7652.

24. **Kalpana, G. V., S. Marmon, W. Wang, G. R. Crabtree, and S. P. Goff.** 1994. Binding and stimulation of HIV-1 integrase by a human homolog of yeast transcription factor SNF5. Science **266:**2002-2006.

25. **Kamihira, M., K. Ono, K. Esaka, K. Nishijima, R. Kigaku, H. Komatsu, T. Yamashita, K. Kyogoku, and S. Iijima.** 2005. High-level expression of single-chain Fv-Fc fusion protein in serum and egg white of genetically manipulated chickens by using a retroviral vector. J. Virol. **79:**10864-10874.

26. **Kulkosky, J., K. S. Jones, R. A. Katz, J. P. G. Mack, and A. M. Skalka.** 1992. Residues critical for retroviral integrative recombination in a region that is highly conserved among retroviral/retrortansposon integrases and bacterial insertion sequence transposases. Mol. Cell. Biol. **12:**2331-2338.

27. **Lai, L., H. Liu, X. Wu, and J. C. Kappes.** 2001. Moloney murine leukemia virus integrase protein augments viral DNA synthesis in infected cells. J. Virol. **75:** 11365–11372.

28. **Lee, J.-S., K. M. Galvin, R. H. See, R. Eckner, D. Livingston, E. Moran, and Y. Shi.** 1995. Relief of YY1 transcriptional repression by adenovirus E1A is mediated by E1A-associated protein p300. Genes Dev. **9:**1188-1198.

29. **Lee, J.-S., K. M. Galvin, and Y. Shi.** 1993. Evidence for physical interaction between the zinc-finger transcription factors YY1 and Sp1. Proc. Natl. Acad. Sci. USA **90:**6145-6149.

30. **Lewinski, M. K., M. Yamashita, M. Emerman, A. Ciuffi, H. Marshall, G. Crawford, F. Collins, P. Shinn, J. Leipzig, S. Hannenhalli, C. C. Berry, J. R. Ecker, and F. D. Bushman.** 2006. Retroviral DNA integration: viral and cellular determinants of target-site selection. PLoS Pathog. **2**: 611-622.

31. **Lewis, P. F., and M. Emerman.** 1994. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. J. Virol. **68:**510-516.

32. **Li, L., J. M. Olvera, K. E. Yoder, R. S. Mitchell, S. L. Butler, M. Lieber, S. L. Martin, and F. D. Bushman.** 2001. Role of the non-homologus DNA end joining pathway in the early steps of retroviral infection. EMBO J. **20:**3272-3281.

33. **Li, M., and R. Craigie.** 2005. Processing of viral DNA ends channels the HIV-1 integration reaction to concerted integration. J. Biol. Chem. **280:**29334-29339.

34. **Maertens, G., P. Cherepanov, W. Pluymers, K. Busschots, E. De Clercq, Z. Debyser, and Y. Engelborghs.** 2003. LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. J. Biol. Chem. **278:**33528-33539.

35. **Mansharamani, M., D. R. M. Graham, D. Monie, K. K. Lee, J. E. K. Hildreth, R. F. Siliciano, and K. L. Wilson.** 2003. Barrier-to-autointegration factor BAF binds p55 Gag and matrix and is a host component of human immunodeficiency virus type 1 virions. J. Virol. **77:**13084-13092.

36. **Marshall, H. M., K. Ronen, C. Berry, M. Llano, H. Sutherland, D. Saenz, W. Bickmore, E. Poeschla, and F. D. Bushman.** 2007. Role of PSIP1/LEDGF/p75 in lentiviral infectivity and integration targeting. PLoS ONE **2:**e1340.

37. **Mitchell, R. S., B. F. Beitzel, A. R. W. Schroder, P. Shinn, H. Chen, C. C. Berry, J. R. Ecker, and F. D. Bushman.** 2004. Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. PLoS Biol. **2:**e234.

38. **Ono, K., M. Kamihira, Y. Kuga, H. Matsumoto, A. Hotta, T. Itoh, K. Nishijima, N. Nakamura, H. Matsuda, and S. Iijima.** 2003. Production of anti-prion scFv-Fc fusion proteins by recombinant animal cells. J. Biosci. Bioeng. **95:**231-238.

39. **Palko, L., H. W. Bass, M. J. Beyrouthy, and M. M. Hurt.** 2004. The Yin Yang-1 (YY1) protein undergoes a DNA-replication-associated switch in localization from the cytoplasm to the nucleus at the onset of S phase. J. Cell Sci. **117:**465-476.

40. **Petkova, V., M. J. Romanowski, I. Sulijoadikusumo, D. Rohne, P. Kang, T. Shenk, and A. Usheva.** 2001. Interaction between YY1 and the retinoblastoma protein. J. Biol. Chem. **276:**7932-7936.

41. **Puglia, J., T. Wang, C. Smith-Snyder, M. Cote, M. Scher, J. N. Pelletier, S. John, C. B. Jonsson, and M. J. Roth.** 2006. Revealing domain structure through linker-scanning analysis of the murine leukemia virus (MuLV) RNase H and MuLV

and human immunodeficiency virus type 1 integrase proteins. J. Virol. **80:**9497-9510.

42. **Roe, T., T. C. Reynolds, G. Yu, and P. O. Brown.** 1993. Integration of murine leukemia virus DNA depends on mitosis. EMBO J. **12:**2099-2108.
43. **Satijn, D. P. E., K. M. Hamer, J. den Blaauwen, and A. P. Otte.** 2001. The Polycomb group protein EED interacts with YY1, and both proteins induce neural tissue in *Xenopus* embryos. Mol. Cell. Biol. **21:**1360-1369.
44. **Segura-Totten, M., and K. L. Wilson.** 2004. BAF: roles in chromatin, nuclear structure and retrovirus integration. Trends Cell Biol. **14:**261-266.
45. **Serhan, F., M. Penaud, C. Petit, T. Leste-Lasserre, S. Trajcevski, D. Klatzmann, G. Duisit, P. Sonigo, and P. Moullier.** 2004. Early detection of a two-long-terminal-repeat junction molecule in the cytoplasm of recombinant murine leukemia virus-infected cells. J. Virol. **78:**6190-6199.
46. **Shi, Y., J.-S. Lee, and K. M. Galvin.** 1997. Everything you have ever wanted to know about Yin Yang 1……. Biochim. Biophys. Acta **1332:**F49-F66.
47. **Shields, A., O. N. Witte, E. Rothenberg, and D. Baltimore.** 1978. High frequency of aberrant expression of Moloney murine leukemia virus in clonal infections. Cell **14:**601-609.
48. **Shun, M.-C., N. K. Raghavendra, N. Vandergraaff, J. E. Daigle, S. Hughes, P. Kellam, P. Cherepanov, and A. Engelman.** 2007. LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration. Genes Dev. **21**: 1767-1778.

49. **Simon, J. A., and R. E. Kingston.** 2009. Mechanisms of Polycomb gene silencing: knowns and unknowns. Nat. Rev. Mol. Cell Biol. **10:**697-708.

50. **Studamire, B., and S. P. Goff.** 2008. Host proteins interacting with the Moloney murine leukemia virus integrase: multiple transcriptional regulators and chromatin binding factors. Retrovirology **5:**48.

51. **Suzuki, Y., and R. Craigie.** 2007. The road to chromatin-nuclear entry of retroviruses. Nat. Rev. Microbiol. **5:**187-196.

52. **Suzuki, Y., H. Yang, and R. Craigie.** 2004. LAP2αand BAF collaborate to organize the Moloney murine leukemia virus preintegration complex. EMBO J. **23:**4670-4678.

53. **Thomas, M. J., and E. Seto.** 1999. Unlocking the mechanisms of transcription factor YY1: are chromatin modifying enzymes the key? Gene **236:**197-208.

54. **Tsukahara, T., H. Agawa, S. Matsumoto, M. Matsuda, S. Ueno, Y. Yamashita, K. Yamada, N. Tanaka, K. Kojima, and T. Takeshita.** 2006. Murine leukemia virus vector integration favors promoter regions and regional hot spots in a human T-cell line. Biochem. Biophys. Res. Commun. **345:**1099-1107.

55. **Villanueva, R. A., C. B. Jonsson, J. Jones, M. M. Georgiadis, and M. J. Roth.** 2003. Differential multimerization of Moloney murine leukemia virus integrase purified under nondenaturing conditions. Virology **316:**146-160.

56. **Violot, S., S. S. Hong, D. Rakotobe, C. Petit, B. Gay, K. Moreau, G. Billaud, S. Priet, J. Sire, O. Schwartz, J.-F. Mouscadet, and P. Boulanger.** 2003. The human *Polycomb* group EED protein interacts with the integrase of human immunodeficiency virus type 1. J. Virol. **77:**12507-12522.

57. **Wang, C.-Y., Y.-J. Liang, Y.-S. Lin, H.-M. Shih, Y.-S. Jou, and W. C. Y. Yu.** 2004. YY1AP, a novel co-activator of YY1. J. Biol. Chem. **279:**17750-17755.

58. **Wilkinson, T.A., K. Januszyk, M. L. Phillips, S. S. Tekeste, M. Zhang, J. T. Miller, S. F. J. Le Grice, R. T. Clubb, and S. A. Chow.** 2009. Identifying and characterizing a functional HIV-1 reverse transcriptase-binding site on integrase. J. Biol. Chem. **284:**7931-7939.

59. **Woerner, A. M., and C. J. Marcus-Sekura.** 1993. Characterization of a DNA binding domain in the C-terminus of HIV-1 integrase by deletion mutagenesis. Nucleic Acids Res. **21:**3507-3511.

60. **Wu, S., Y. Shi, P. Mulligan, F. Gay, J. Landry, H. Liu, J. Lu, H. H. Qi, W. Wang, J. A. Nickoloff, C. Wu, and Y. Shi.** 2007. A YY1-INO80 complex regulates genomic stability through homologous recombination-based repair. Nat. Struct. Mol. Biol. **14:**1165-1172.

61. **Wu, X., Y. Li, B. Crise, and S. M. Burgess.** 2003. Transcription start regions in the human genome are favored targets for MLV integration. Science **300:**1749-1751.

62. **Wu X., H. Liu, H. Xiao, J. A. Conway, E. Hehl, G. V. Kalpana, V. Prasad, and J. C. Kappes.** 1999. Human immunodeficiency virus type 1 integrase protein promotes reverse transcription through specific interactions with the nucleoprotein reverse transcription complex. J. Virol. **73:** 2126-2135.

63. **Xi, H., Y. Yu, Y. Fu, J. Foley, A. Halees, and Z. Weng.** 2007. Analysis of overrepresented motifs in human core promoters reveals dual regulatory roles of YY1. Genome Res. **17:**798-806.

64. **Yan, N., P. Cherepanov, J. E. Daigle, A. Engelman, and J. Lieberman.** 2009. The SET complex acts as a barrier to autointegration of HIV-1. PLoS Pathog. **5:** e1000327.

65. **Yang, F., O. Leon, N. J. Greenfield, and M. J. Roth.** 1999. Functional interactions of the HHCC domain of Moloney murine leukemia virus integrase revealed by nonoverlapping complementation and zinc-dependent dimerization. J. Virol. **73:**1809-1817.

66. **Yang, W.-M., C. Inouye, Y. Zeng, D. Bearss, and E. Seto.** 1996. Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. Proc. Natl. Acad. Sci. USA **93:**12845-12850.

67. **Yang, W.-M., Y.-L. Yao, J.-M. Sun, J. R. Davie, and E. Seto.** 1997. Isolation and characterization of cDNAs corresponding to an additional member of the human histone deacetylase gene family. J. Biol. Chem. **272:**28001-28007.

68. **Yao, Y-L., W.-M. Yang, and E. Seto.** 2001. Regulation of transcription factor YY1 by acetylation and deacetylation. Mol. Cell. Biol. **21:**5979-5991.

69. **Yung, E., M. Sorin, E.-J. Wang, S. Perumal, D. Ott, and G. V. Kalpana.** 2004. Specificity of interaction of INI1/hSNF5 with retroviral integrases and its functional significance. J. Virol. **78:**2222-2231.

70. **Zheng, R., T. M. Jenkins, and R. Craigie.** 1996. Zinc folds the N-terminal domain of HIV-1 integrase, promotes multimerization, and enhances catalytic activity. Proc. Natl. Acad. Sci. USA **93:**13659-13664.

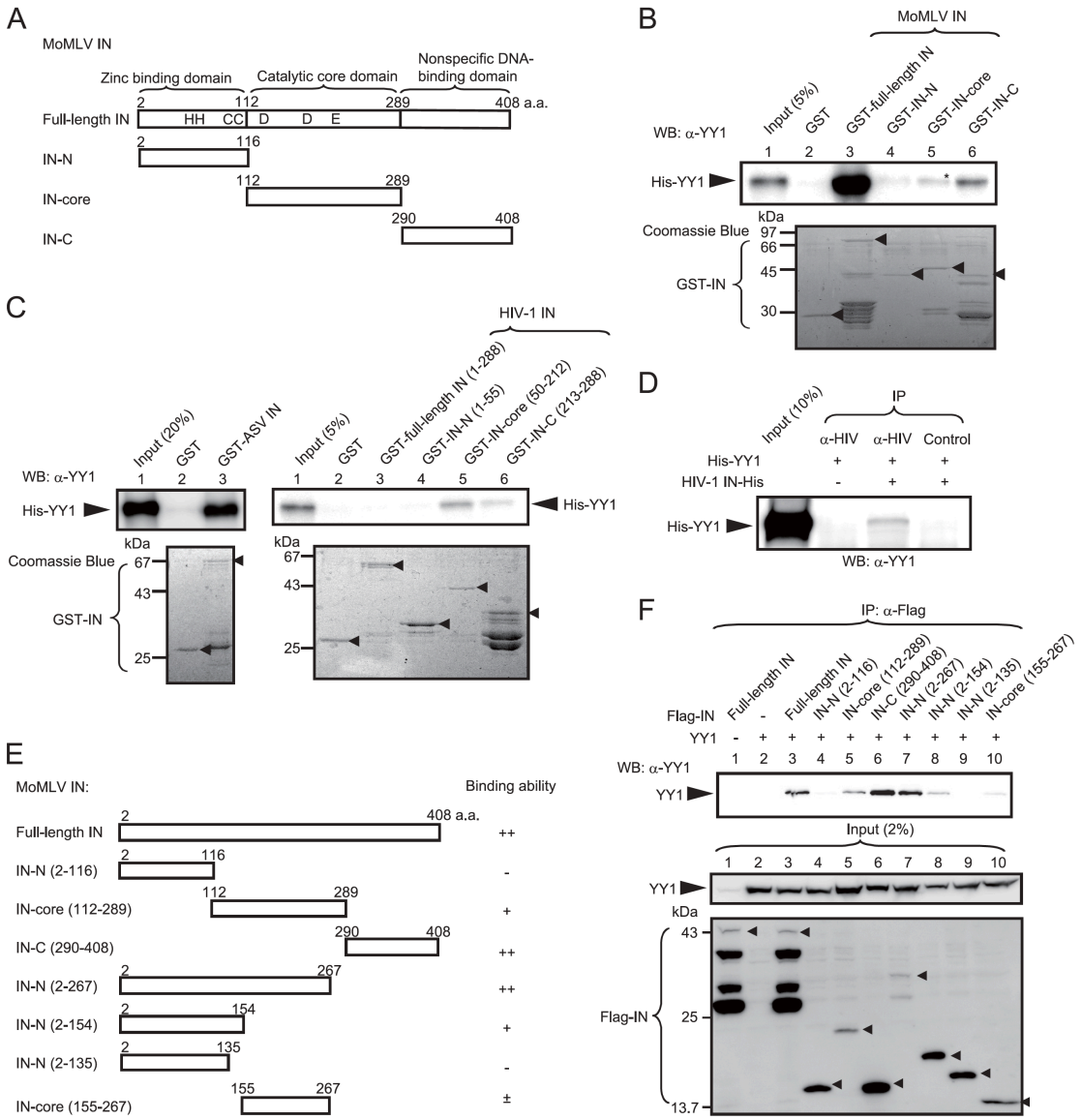

FIG. 1. YY1 directly interacts with INs of MoMLV, ASV and HIV-1. (A) A schematic drawing of the GST-IN of MoMLV and its deletion mutants. HHCC: zinc-finger, DDE: catalytic center. (B) In vitro binding assay of GST-MoMLV IN derivatives and His-YY1. His-YY1 was detected with the anti-YY1 antibody (upper panel). The GST fusion proteins used for the pull-down assay were subjected to SDS-PAGE and detected by Coomassie brilliant blue (CBB)-staining (lower panel).

Arrowheads indicate the intact fragments of the GST-IN derivatives. A representative result of several experiments is presented. Band intensity was measured using a densitometer and faint bands of lane 4 and 5 were analyzed by paired *t*-test. Asterisk indicates significance of GST-IN-core (lane 5) values versus GST values (lane 2) (*, $P = 0.018$). (C) In vitro binding assays of GST-ASV IN and GST-HIV-1 IN with His-YY1. The amino acid number of HIV-IN is taken from the NCBI database (NC_001802). (D) Immunoprecipitation of purified His-HIV-1 IN and His-YY1. Five hundred nanogram each of His-HIV-1 IN and His-YY1 were mixed in 300 ml binding buffer used for pull-down assay, the immunocomplex was precipitated with anti-HIV-1 IN antiserum and analyzed with the anti-YY1 antibody. For a negative control, mouse pre-immune serum was used. (E) A schematic drawing of the Flag-tagged MoMLV IN and its deletion mutants. Full-length MoMLV IN and the mutants were tagged with Flag at the N-terminus. The result of immunoprecipitation experiment is summarized on the right side of the panel. (F) Human YY1 interacts with the catalytic core and C-terminal domain of MoMLV IN. Cells were lysed with buffer B. YY1 precipitated with ANTI-FLAG M2 affinity beads was detected with anti-YY1 antibody after extensive washing with buffer B. Two percent of each sample was used to confirm the expression of YY1 or Flag-INs (input, lower panels). The intact bands of the Flag-IN fragments are indicated by the arrowheads. A representative result of several experiments is presented.

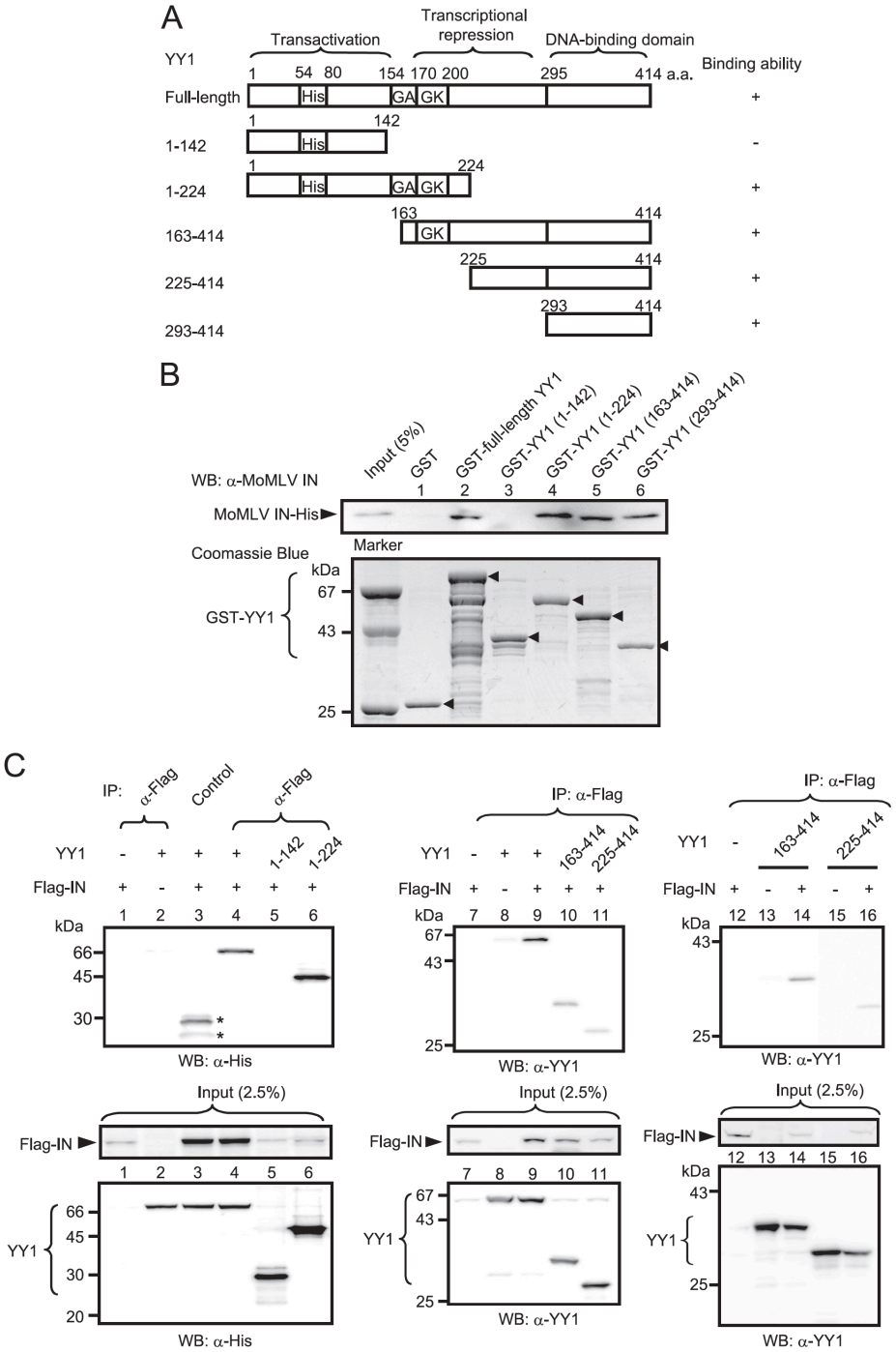

FIG. 2. Identification of the IN-binding region in YY1 in vitro and in vivo. (A) A schematic drawing of full-length YY1 and its fragments used for pull-down and co-immunoprecipitation experiments. His, histidine-rich domain; GA,

glycine-alanine–rich domain; GK, glycine-lysine–rich domain. The results of in vitro pull-down and immunoprecipitation experiments are summarized on the right side of the panel. (B) Pull-down assay of MoMLV IN with GST fusion proteins of various portions of the human YY1. His-MoMLV IN was detected by western blotting using anti-MoMLV IN antiserum. GST and GST-YY1 fragments were subjected to SDS-PAGE and the gel was stained with CBB. Arrowheads show the intact fragments of GST-YY1 derivatives (lower panel). A representative result of several experiments is presented. (C) Co-immunoprecipitation of human YY1 and MoMLV IN expressed in 293FT cells (upper panels). Normal mouse IgG was used in the control, and nonspecific bands are indicated by asterisks. Cells were lysed with buffer A. IN complex was precipitated and washed with buffer A (left and central panels) or buffer B (right panel). In the right panel, IN minus controls were included to estimate a non-specific interaction between YY1 and anti-Flag antibody observed in lane 8, since interaction between IN and some YY1 derivatives seemed to be weak. His-tagged fragments of human YY1 were detected with anti-YY1 or anti-His antibody. These antibodies were also used for detection of input YY1 derivatives (2.5%). Flag-IN was detected with anti-Flag antibody. A representative result of several experiments is presented.

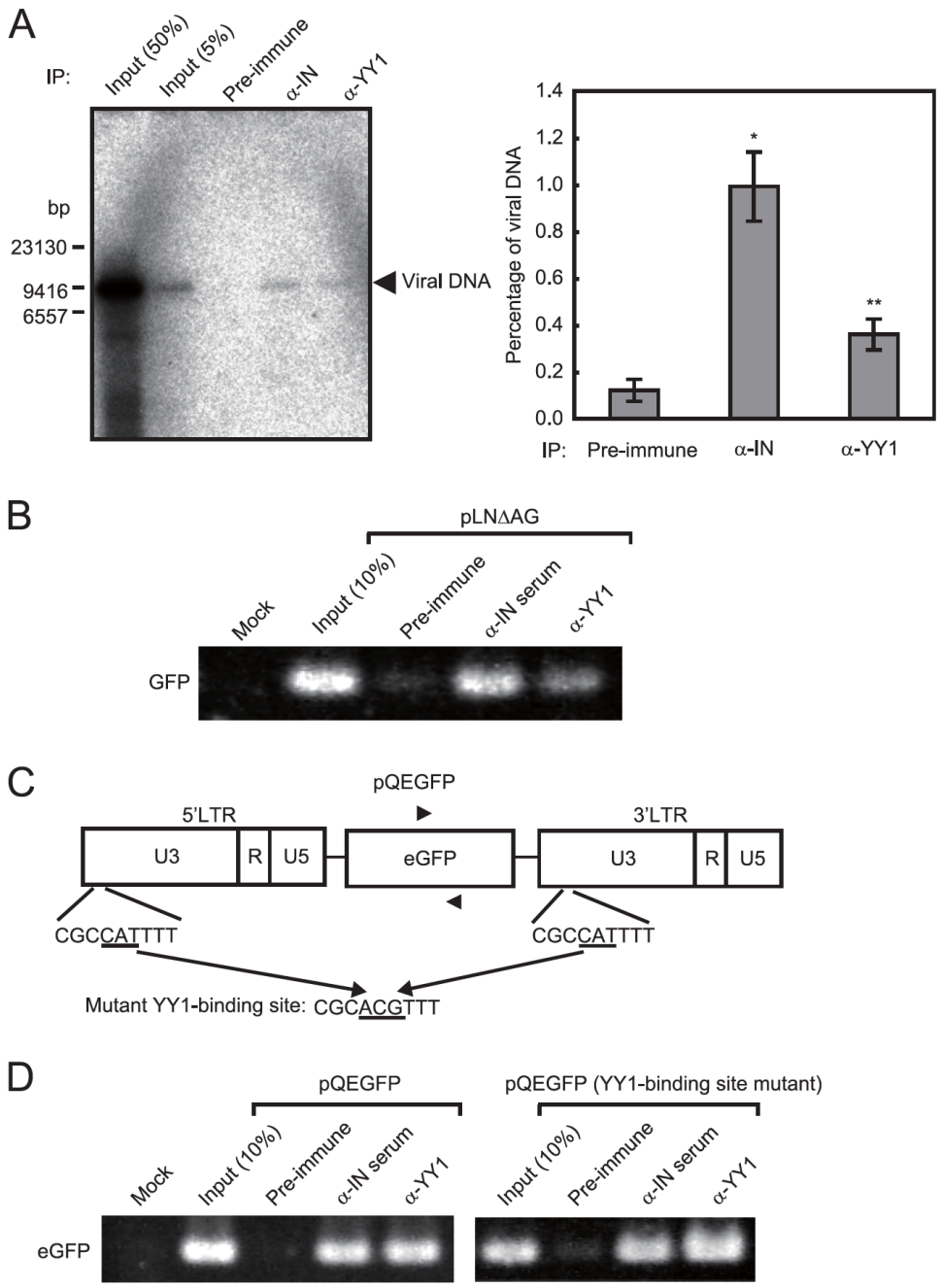

FIG. 3. YY1 associates with MoMLV cDNA in infected cells. (A) The cytoplasmic fraction containing PICs was prepared from co-cultures of NIH3T3 and clone #4 cells, and PICs were partially purified by gel filtration, and immunoprecipitated with anti-MoMLV IN antiserum or anti-YY1 antibody. Pre-immune rabbit serum was used

as a control antibody, and the viral sequence was detected by Southern blotting using a probe for the LTR of MoMLV. The right panel shows percentage of immunoprecipitated viral DNA. Immunoprecipitated viral DNA was quantitated by qPCR using primers for packaging signal sequence; 5'-CGCTCACAACCAGTCGGTAGAT-3' and 5'-AGGTCACGATGTAGGGGACCT-3'. Asterisks indicate that values of anti-IN and anti-YY1 compared to those of pre-immune serum were significantly different (*, P = 0.009, **, P = 0.046). (B) ChIP assay of NIH3T3 cells infected with the VSV-G-pseudotyped MoMLV vector pLNΔAG (MOI of 0.8). Infected cells were harvested 4 h post-infection. Whole cell lysates were subjected to the assay. (C) A schematic representation of the YY1-binding site mutation in the LTR. Primers used in Fig. 3D are shown by the arrowheads. (D) The YY1-binding sequences in the LTRs do not affect the association of YY1 to viral DNA. GP293 cells were co-transfected with pVSV-G and the YY1-binding site mutant pQEGFP to obtain the viral vector. NIH3T3 cells were infected with the wild type or mutant viral vector at the same MOI (1.6), and the cells were collected 4 h post-infection. PICs were immunoprecipitated from the cytoplasmic fraction with anti-MoMLV IN serum or anti-YY1 antibody. Pre-immune rabbit serum was used as a control antibody. PCR was performed to detect the eGFP sequence of the viral vector.

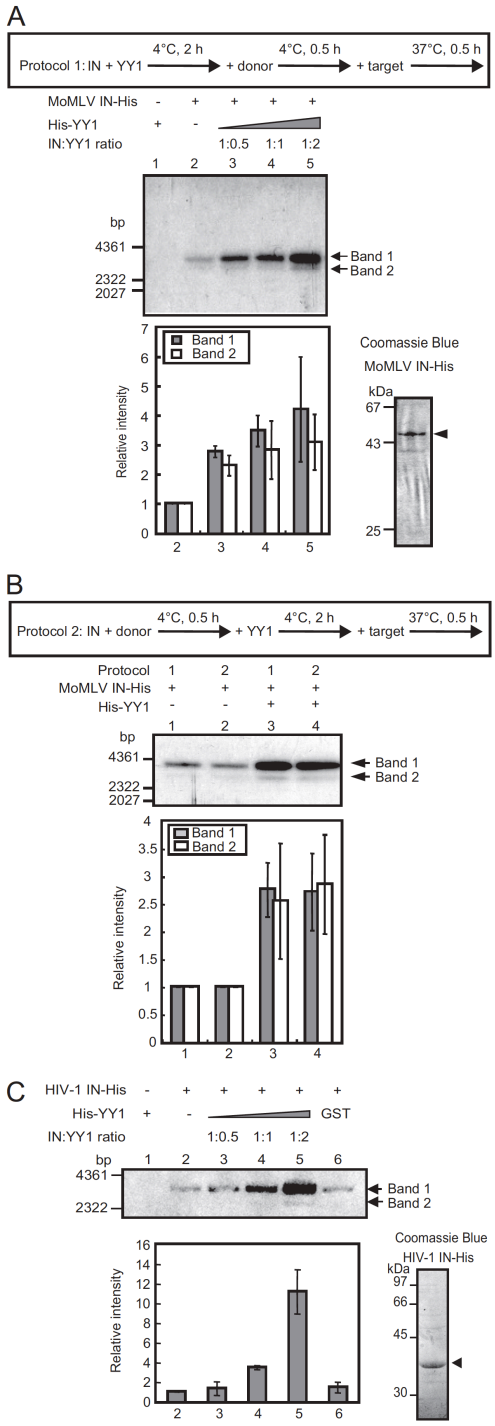

FIG. 4. YY1 enhances integration activity of MoMLV and HIV-1 INs. The purity of the IN used is presented in the photographs on the right side. (A) Following protocol 1, His-YY1 was mixed with MoMLV IN first at a molar ratio as described above the panel.

The upper panel shows the products from the ^{32}P-labeled LTR donor and the target plasmid DNA by integration. The lower bar graph indicates the normalized band intensity of the integration products compared to that of the IN plus GST (lane 2) as 1. The band density was measured using an image analyzer (BAS-5000, Fujifilm). Error bars show the standard deviation of three independent assays. (B) Comparison of the activation of MoMLV IN by YY1 following protocols 1 and 2 at a ratio of IN and YY1 of 1:2. The lower bar graph indicates the normalized band intensity of the integration products compared to that of the IN plus GST (lanes 1 and 2) as 1. (C) YY1 also activated HIV-1 IN. In lane 6, GST (20 pmol) was included to quantify non-specific activation. The bar graph shows the normalized band intensity of the products (Band 1).

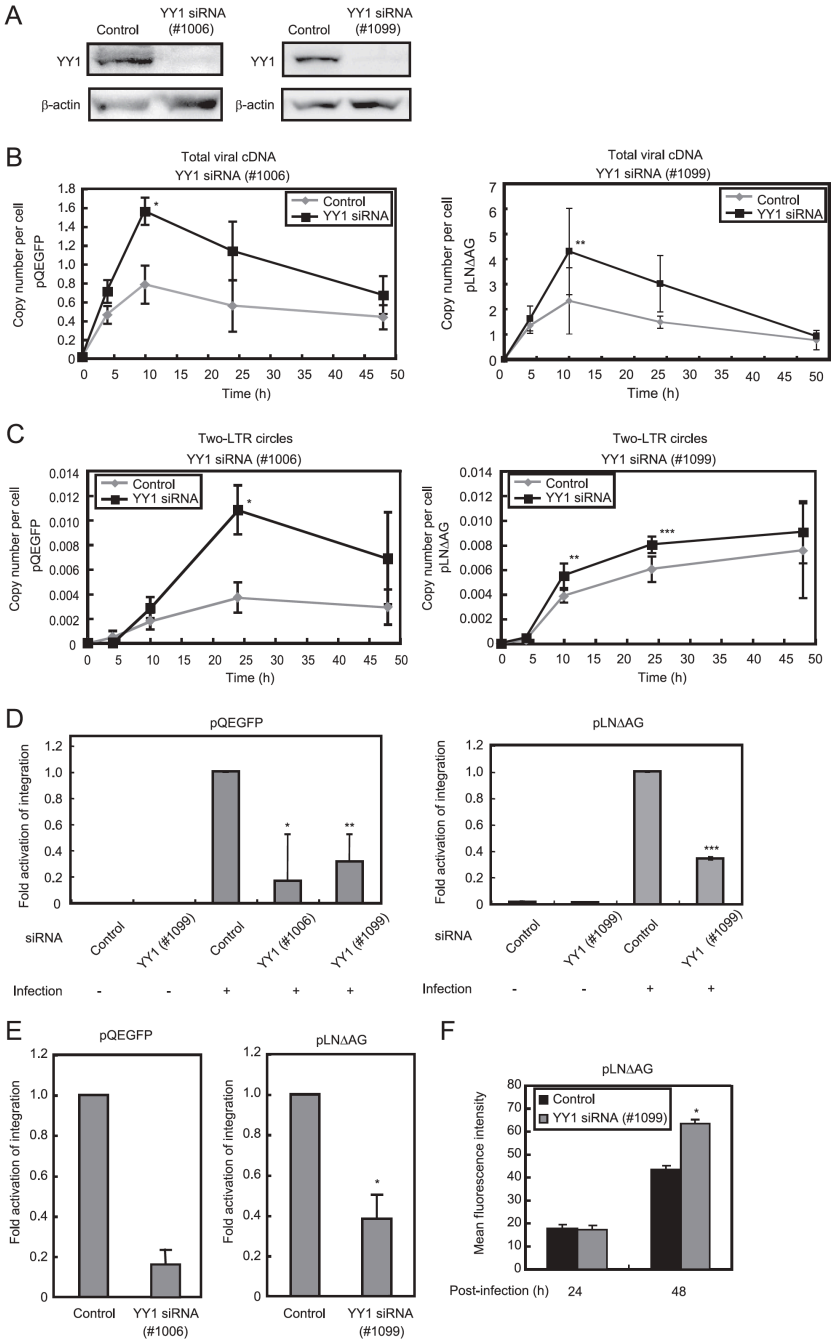

FIG. 5. YY1-knockdown reduces viral integration into host genome. (A) Knockdown of YY1 by siRNA was confirmed by western blotting. The amount of total viral cDNA (B) and two-LTR circle (C) of pQEGFP (siRNA1006) and pLNΔAG (siRNA1099) viral vectors. NIH3T3 cells were transfected twice with YY1 siRNA

#1006 or #1099, and then infected with pQEGFP (MOI of 0.1) or pLNΔAG (MOI of 1). Cellular DNA was extracted at 0, 4, 10, 24 and 48 h and subjected to qPCR analysis. Copy number per cell was calculated by normalizing the value of the viral sequence with that of GAPDH. Error bars represent the standard deviation of at least three experiments. Primers used for PCR were GAPDH: 5'-TGTGATGGGTGTGAACCACGAGAA-3' and 5'-GAGCCCTTCCACAATGCCAAAGTT-3'. Asterisks indicate that values of YY1 siRNA compared to those of scramble RNA were significantly different (*, $P = 0.038$, **, $P = 0.031$ in (B), *, $P = 0.022$, **, $P = 0.041$, ***, $P = 0.047$ in (C)). (D) The integrated form of viral DNA was quantified 14 days post-infection by qPCR and normalized as described for the results presented in Fig. 5C. Asterisks indicate significance of values of YY1 siRNA versus those of scramble RNA (*, $P = 0.014$, **, $P = 0.016$, ***, $P < 0.0001$). (E) The integrated viral DNA was quantified by nested PCR 24 h post-infection (pQEGFP: n = 2, pLNΔAG: n = 3). Asterisk indicates that values of YY1 siRNA compared to those of scramble RNA were significantly different (*, $P = 0.027$). (F) Triplicated cultures of NIH3T3 cells (YY1 siRNA and scramble RNA treated) infected with pLNΔAG (MOI of 1) were analyzed by fluorescence-activated cell sorting in order to quantify the mean GFP intensity. Asterisk indicates that values of YY1 siRNA compared to those of scramble RNA were significantly different (*, $P = 0.008$).

TABLE 1. Sequencing results of LTR-target junctions

Duplication	No. (%) of products obtained with:	
	IN	IN+YY1
4 bp	13 (77)	10 (71)
5 bp	2 (12)	2 (14)
3 bp	1 (6)	0 (0)
Others	1 (6)	2 (14)
Total	17	14

Chapter 3

Moloney murine leukemia virus integrase and reverse transcriptase interact with PML proteins

Introduction

In the early stage of the retroviral life cycle, a reverse transcription complex (RTC) is assembled after the entry of retroviral particles into cells, and viral complementary DNA (cDNA) is synthesized. The RTC contains several viral proteins such as integrase (IN), reverse transcriptase (RT) and capsid in Moloney murine leukemia virus (MoMLV) (*1*). Newly synthesized viral cDNA, IN and various viral and cellular proteins then form preintegration complex (PIC) and the complex is imported into the nucleus and viral double stranded cDNA is integrated into the host chromosome (*1*). The precise mechanism by which the RTC transforms into PIC remains to be elucidated (*2*), although detailed analyses of the mechanism of integration have been conducted (*3, 4*). PIC associates with various cellular proteins depending on virus type. For instance, PIC of human immunodeficiency virus type 1 (HIV-1) contains lens epithelium-derived growth factor (LEDGF/p75) (*5, 6*), and promyelocytic leukemia (PML) protein (*2, 7*). MoMLV PIC contains lamina-associated polypeptide 2α (Lap2α) (*8*) and possibly the transcription factor Ying Yang 1 (YY1) (*9*). It is well established that IN itself physically interacts with various proteins (*10*).

The PML body is a distinct structure mainly observed in the nucleus, and PML proteins are key organizers of the body proposed to regulate a variety of cellular

processes including the sequestration, activation or degradation of partner proteins (*11-13*). The PML body recruits an ever-increasing number of proteins, and many of the proteins found in the PML body are modified by small ubiquitin-related modifier (SUMO) (*14*). It has been supposed that non-covalent interaction between the SUMO moiety and SUMO-interacting motif (SIM) is crucial to the formation of the PML body and partly contributes to the recruitment of partner proteins to the body (*11-13*). Recently, SUMOylation of HIV-1 IN has been reported but the physiological importance of the modification has not been clear (*15*). In the present paper, we also found the SUMOylation of MoMLV IN and that IN and RT interacted with PML proteins. We describe the physiological importance of the interaction between these viral proteins and PML proteins.

Materials and Methods

Cell culture

In this study, we used five cell lines including NIH3T3, Hela, clone no. 4 (*16*), 293FT and GP293. The former three cell lines were cultured at 37˚C in atmosphere containing 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). The latter two cell lines were cultured in DMEM supplemented with 10% FCS, 1 mM sodium pyruvate and Non-Essential Amino Acids (Invitrogen).

Vector construction

phCMV2 (Genlantis) was used as an expression vector for FLAG-tagged (phCMV2/FLAG-MLV IN) or HA-tagged (phCMV2/HA-MLV IN) MoMLV IN. As a template for PCR, pFLAG/MLV IN was used (*9*). For the construction of FLAG-tagged RT, the RT sequence was amplified by PCR with the pGP plasmid in a retrovirus packaging kit (Takara) as the template, and 5'-AAAGAATTCATGACCCTAAATATAGAAGATGAGCATCGGCTA-3' and 5'-AAAAAGCTTGAGGAGGGTAGAGGTGTCTGGAGT-3' as primers. The amplified DNA was cloned in pETBlue-2 (Novagen), and then recloned into pcDNA4/HisC derivative containing the FLAG tag (Invitrogen). SUMO-1, SUMO-2 and SUMO-3 sequences were amplified by PCR using cDNA from 293FT cells as the template and the following primers, SUMO-1: 5'-CAT<u>GGATCC</u>TCTGACCAGGAGGCAAAACC-3', 5'-CAT<u>CTCGAG</u>CTAAACTGTTGAATGACCCC-3'; SUMO-2:

5'-CATGGATCCGCCGACGAAAAGCCCAAGGA-3',

5'CATCTCGAGCTGGAGTAAAGAAGCAGGTTC-3'; SUMO-3:

5'-CATGGATCCTCCGAGGGGAGAAGCCCAAGGA-3',

5'-CATCTCGAGCTAGAAACTGTGCCCTGCC-3,' (underlines show a *Bam*HI or *Xho*I site). The amplified DNA was ligated to the HA-tag sequence and cloned to pcDNA4/His.

For the construction of the PML VI-expression vector, PML VI cDNA was amplified from cDNA of NIH3T3 cells using

5'-CATGGATCCGAAACTGAACCAGTTTCCGTG-3' and

5'-CATGCGGCCGCCTGTTTCCCCTGTGTCACT-3' (*Bam*HI and *Not*I sites are underlined). The amplified DNA was introduced into pcDNA4/HisC and pGEX-6P-2 (GE Healthcare). The FLAG-tagged (pFLAG/mPIASy) and non-tagged (pcDNA4/mPIASy) PIASy-expression plasmids were constructed from pSPORT6/mPIASy (*17*).

Knockdown of PML

siRNA: 5'-AAUUCCUCCUGUAUGGCUUGCUCUG-3' was used. NIH3T3 cells (4.0×10^4 per 35-mm dish) were cultured for 24 h then siRNA was introduced at a final concentration of 80 nM by Lipofectamine 2000™ (Invitrogen). After incubation for another 24 h, siRNA was transfected again. Cells were then cultured for 24 h and infected with a MoMLV-based viral vector pQEGFP (*9*). DNA was isolated by using a QIAamp DNA mini kit (Qiagen), and viral cDNA and two-LTR circle viral cDNA were quantified by real-time PCR, and integrated cDNA was determined by nested PCR as

reported (*9*). Rescue experiments were performed by simultaneously introducing the PML VI-expression vector and siRNA.

Immunoprecipitation

To investigate the interaction of endogenous PML with MoMLV IN and RT, the expression plasmid for FLAG-tagged MoMLV IN or FLAG-tagged MoMLV RT was transfected into 293FT cells using Lipofectamine 2000™. Cells were then harvested between 36 and 48 h posttransfection, and lysed in IP lysis buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 0.5% sodium deoxycholate and 1% NP-40). After the cell lysates had been incubated with ANTI-FLAG M2 affinity gel (Sigma) for 4 h, the gel was washed twice with the lysis buffer. The gel was treated twice with 100 U/ml DNase I, 10 μg/ml RNase A and 200 U/ml Benzonase (a broad spectrum endonuclease from *Serratia marcescens*, Novagen) in 20 mM Tris-HCl (pH 7.3), 50 mM KCl and 40 mM $MgCl_2$ at 37°C for 10 min, and washed twice with the lysis buffer. The samples were analyzed by Western blotting using anti-PML antibody (H-238). Anti-FLAG and anti-PML antibodies were purchased from Wako and Santa Cruz, respectively. For confirmation of the SUMOylation of IN, the FLAG-tagged IN expression plasmid was introduced into 293FT cells together with expression plasmids for HA-tagged SUMO-1, -2 or -3 and PIASy. Cells were lysed in 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 500 mM NaCl, 0.5% sodium deoxycholate, 0.5% SDS and 0.5% NP-40 (*18*), and the lysates were heated at 100˚C for 5 min, then IN was immunoprecipitated with M2 affinity beads. SUMOylation of IN was estimated by Western blotting using anti-HA (3F10) (Rosch).

To analyze whether IN and PML interact each other during virus infection, NIH3T3 cells and MoMLV-producer clone no. 4 (*16*) were cocultured in DMEM containing 10% FCS. NIH3T3 and the producer cells were inoculated at a ratio of 3:1. The cocultured cells were lysed with the lysis buffer and supplied for immunoprecipitation using anti-MoMLV IN antiserum (*9*).

In vitro binding assay

To study whether IN or RT physically interacts with the PML protein, purified proteins were prepared. His-tagged IN and GST-tagged full-length, N-terminal, core catalytic, and C-terminal portions of IN were produced in *E. coli* BL21(DE3) or BL21, and purified as described previously (*9*). *E. coli* BL21 containing the GST-tagged PML VI-expression vector was grown in LB medium to an optical density at 600 nm of 0.5. Then 1 mM isopropyl b-D-thiogalactoside was added, and *E. coli* was cultured at 37˚C for 3 h. The cells were suspended in phosphate buffered saline (PBS) containing 300 mM NaCl and disrupted by sonication. For the purification of the GST-tagged PML VI, glutathione-Sepharose 4B (GE healthcare) was used. After the purification, samples were dialyzed against PBS containing 300 mM NaCl. FLAG-tagged RT and His-tagged PML VI were produced in 293FT cells. For the purification of these proteins, M2 affinity beads or TALON metal affinity resin (Clontech) was used under denaturing conditions (20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% NP-40 and 0.5% SDS) to remove associated proteins. After the purification, samples were dialyzed against 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate and 1% NP-40. GST-tagged MoMLV IN and its truncated forms (125-250

ng) were incubated with glutathione-Sepharose 4B in the lysis buffer. Cell lysates were then mixed with the immobilized GST-IN fusion proteins on the beads, and the mixture was allowed to stand for 4 h at 4°C. The beads were washed three times with the lysis buffer, treated with a mixture of Benzonase, DNase I and RNase A, and then mixed with SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, 2% SDS, 5% sucrose and 0.002% bromophenol blue). The bound proteins underwent SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting using anti-PML antibody. Pulldown assay using GST-tagged PML VI and His-tagged IN, and *in vitro* immunoprecipitation assays using His-tagged PML VI and FLAG-tagged RT were also performed.

Immunocytochemical analyses

Hela cells were transfected with the FLAG-tagged IN expression vector with or without HA-tagged SUMO-1 and PIASy expression vectors and cultured on coverslips. The cells were rinsed with cold PBS three times, fixed with freshly prepared 4% paraformaldehyde for 30 min at room temperature, washed three more times in PBS, and permeabilized with PBS containing 0.5% Triton X-100 for 30 min at room temperature. After another three washes with PBS, the cells were incubated with PBS containing Block Ace™ powder (DS Pharmabiomedical) for 1 h at room temperature. The coverslips were rinsed with PBS three times and dipped in primary antibodies in PBS for 1 h at room temperature. The primary antibodies used were rabbit anti-PML and mouse anti-FLAG antibodies. After being rinsed with cold PBS three times, cells were incubated with goat anti-mouse IgG coupled to fluorescein isothiocyanate and goat

anti-rabbit IgG coupled to tetramethylrhodamin-isothiocyanate (ZyMax^{TM}, Zymed Laboratories) for 30 min at room temperature. After three more washes with PBS, the coverslips were mounted onto glass slides with 90% glycerol and 10% PBS. Samples were analyzed using a laser scanning microscope (Olympus FV300 and FV1000).

Results

Interaction between MoMLV IN and PML

To clarify the host-viral interaction with MoMLV, we screened cellular proteins that interact with IN as reported previously (*9*) and found that the PML protein interacted with MoMLV IN in transient expression and coimmunoprecipitation experiments. As shown in Fig. 1A, immunoprecipitation was performed with cells in which FLAG-tagged IN was transiently expressed. For PML, it is well known that a variety of carboxy-terminal domains generated by alternative splicing yield isoforms (*11*). Several endogenous PML isoforms such as PML I/II, III/IV, V, VI and VII were detected in the immunocomplex and PML VI gave the most intense band among them. These bands were estimated to be each isoform based on their molecular weight and a recent report describing human PML (*19*). However, several proteins detected in the immunocomplex could not be identified (marked by asterisks in Fig. 1A). Since SUMO-conjugation of the PML protein is crucial for the PML-body's formation, the blot was reprobed with anti-SUMO antibody. However, SUMOylated PML was not detected in the immunocomplex. Under the condition, SUMOylated IN was not detected either.

We then checked whether IN interacts with PML protein in the viral context. For this, MoMLV-producing cells were cocultured with normal NIH3T3 cells (*9*) and the interaction was studied by coimmunoprecipitation. As shown in Fig. 1B, PML protein, possibly isoform VI judging from their molecular mass could be detected in the

immunocomplex precipitated by anti-MoMLV IN antibody. We also could detect PML I/II isoforms in this experiment (data not shown). This result is reasonable since PIC contains PML (*2, 7*).

SUMOylation of MoMLV IN

Generally, it has been reported that the binding between SUMO and SIM of PML protein is crucial for the PML-body's formation (*20*), and PML-body-associated proteins are recruited to the body through SUMO-SIM interaction or SUMO-independent interaction between PML and the partner proteins (*11-13*). Furthermore, it has recently been reported that HIV-1 IN is potentially SUMOylated (*15*). Therefore, we studied whether MoMLV IN is SUMOylated, although we did not detect a SUMO-conjugated form of IN in the transient expression and immunoprecipitation experiments shown in Fig. 1A. As shown in Fig. 2A, one of the SUMO-E3 ligases PIASy interacted with MoMLV IN and the IN protein was SUMOylated in the presence of exogenous SUMOs and PIASy (Fig. 2B). In the Fig. 2B, we performed immunoprecipitation under the denaturing condition. Bands marked by asterisks in lanes 9-11 of the middle left panel seemed to be consistent with mono-SUMOylated INs judging from their molecular weight (around 57 kD), although the bands could not be detected by anti-FLAG because of the presence of heavy chain (upper left). Putative oligo-SUMOylated bands were also detected by anti-HA antibody. Sometimes, SUMO-E3 ligase tightly bound to its substrate. However, under the condition, PIASy was not precipitated in the immunocomplex. Therefore, bands

detected by anti-HA were not SUMOylated PIASy. Either SUMO-1, -2, or -3 seemed to be conjugated with IN. We also studied another E3 ligase, Pc2 (*21*), but could not detect any SUMO-conjugated IN (data not shown). Although we could not rule out the possibility that other E3 ligase catalyzes the SUMOylation of IN, PIASy is a candidate for IN-E3 ligase.

IN physically interacts with PML

In Fig. 3A, GST pulldown was performed with *E. coli*-produced full-length, N-terminal, catalytic core and C-terminal portions of IN (*9*) and the PML protein produced by cultured cells. The results of the pulldown indicated that PML VI mainly bound to IN, although the degradation of GST-IN was evident. This result did not rule out the possibility that other isoforms of PML bound to IN because of the low detection level of protein under the conditions. In fact, with a longer exposure, we could detect other PML isoform, possibly PML I/II. Then, we reprobed the blot with anti-SUMO-1 antibody but could not detect SUMO modification. We also tried pulldown assays using cell extracts from SUMO-1 knockdown cells and cells that transiently expressed SUMO-1 and PIASy. However, obvious difference of binding between IN and PML was not observed (data not shown). We could not clarify whether the interaction between IN and PML was based on SUMO-SIM interaction or SUMO independent interaction from these experiments, since detection of SUMO-conjugated PML protein itself was difficult under our experimental condition due to low sensitivity of anti-SUMO-1 antibody we used, or low abundance of SUMOylated PML species.

Furthermore, the result of pulldown assay using truncated INs showed that catalytic core and C-terminal portions of IN played an important role in the interaction (Fig. 3A). We then performed the pulldown experiment with purified PML VI protein (Fig. 3B). In the experiment, GST-fused PML VI expression vector was introduced into *E. coli* BL21, and PML protein was purified. PML VI interacted with His-tagged IN, indicating again that IN directly bound to PML VI protein.

Localization of IN and PML

We then studied the subcellular localization of transiently expressed IN by using laser scanning microscopy. As shown in Fig. 4A, IN was detected as speckle-like structures with the FITC-labeled antibody and the PML protein was also detected as speckles with the TRITC-conjugated antibody. Merged color (yellow) indicated colocalization of IN and PML proteins, suggesting that IN may be included in the PML body. We also confirmed that the speckle-like structures localized within nuclei by DAPI staining (Fig. 4B). When PIASy alone or PIASy plus HA-SUMO-1 were expressed together with FLAG-IN, IN was also detected as speckles, and the size and numbers of the speckle structure seemed not to be significantly affected (Fig. 4A).

Function of PML in MoMLV infection

To know the physiological function of the PML protein in the viral life cycle, PML was knocked down by siRNA in NIH3T3 cells, and then, a VSV-G-pseudotyped

retroviral vector (pQEGFP) was introduced with a multiplicity of infection of 0.5-1.0. We analyzed viral cDNA synthesis and integration efficiency as well as two-LTR circle viral cDNA formation that roughly reflects the nuclear entry of PICs (*9*). As shown in Fig. 5A, the amount of PML protein was reduced by around 20% of untreated cells after two cycles of siRNA transfection. The amounts of total viral cDNA and two-LTR circle viral cDNA were determined after the infection. In PML-knockdown cells, the amounts of viral cDNA decreased less than 50% of the control after 10 h postinfection (Fig. 5B), and two-LTR circle cDNA was about 60% of the control (Fig. 5C). The amount of integrated viral cDNA was almost 50% of the control in PML-knockdown cells (Fig. 5D). Furthermore, we performed rescue experiments by introducing expression vector for PML VI together with siRNA. As shown in Fig. 5E, PML VI expression gave higher cDNA production compared to the control suggesting again the positive effect of PML protein on the cDNA production. Totally, these results suggested that the reduction in two-LTR circle cDNA formation and integration by the siRNA might be a simple reflection of the reduced reverse transcription. For 5 days after the viral infection, growth retardation possibly by siRNA was not observed.

RT also interacts with PML

Since RT seemed to be activated by PML proteins, we investigated their physical interaction. FLAG-tagged RT was transiently expressed in 293FT cells and immunoprecipitated by the anti-FLAG antibody. As shown in Fig. 6A, endogenous PML proteins were precipitated with FLAG-tagged RT. This result indicated that RT

also interacted with PML proteins. PML VI was mainly precipitated by the anti-FLAG antibody. The interaction was also confirmed by the *in vitro* binding assay. For this, purified FLAG-tagged RT and purified His-tagged PML VI protein were used (Figs. 6B and 6C). The purity of these proteins was confirmed by silver staining (data not shown).

Discussion

In previous reports, PML and INI1 were recruited to cellular PIC and RTCs upon retroviral infection (*2, 7*). In the present study, we confirmed that IN and PML proteins interacted physically, which may contribute to the recruitment of PML proteins to PIC. We also found that RT interacted with PML, which also supports the finding that the RTC contains PML (*2*). *In vivo*, PML proteins activated the RT reaction. We could assume two mechanisms for this activation. First, interaction with PML directly affected RT activity. Second, IN facilitates the RT reaction through physical interaction as reported previously (*22, 23*), and PML modulates this interaction. In fact, we reported a similar but opposite influence of YY1; IN-YY1 interaction may inhibit RT (*9*). It is also possible that SUMOylation of IN affects this interaction.

It was reported that HIV-1 IN is SUMOylated, and SUMO conjugation facilitates a step of the early viral life cycle between after reverse transcription and before integration. In the report, SUMO-1, -2, and -3 all conjugated to IN (*15*). In the present paper, we found that MoMLV IN was SUMOylated, possibly by the actions of PIASy as an E3 ligase. Transient expression of PIASy and SUMO proteins in cultured cells showed that SUMO-1, -2, or -3 conjugated MoMLV IN could be detected. A previous report demonstrated that MoMLV capsid is SUMOylated by PIASy and that the SUMOylation of capsid is important between after reverse transcription and before nuclear entry (*24*). Thus, it is rational that SUMOylation of IN is catalyzed by PIASy.

We found that exogenously expressed IN colocalized with PML proteins in speckle-like structures. In the previous report, overexpressed HIV-1 IN was reported to

localize in the nuclei (*15*). Zhang et al. also reported that overexpressed HIV-1 IN was colocalized in a nuclear structure with TTRAP, one of the proteins detected in the PML body. Furthermore, they indicated that TTRAP facilitates integration of viral cDNA in the host chromosome (*25*). Together, these results including ours suggested that transiently expressed IN might localize in the PML body. Overexpressed proteins often accumulate in the PML body possibly for degradation (*12*). Thus, we cannot approve the physiological meaning of the localization in the PML body, although IN interacted with PML proteins in a viral context and PML possibly activated RT.

For the PML-body's formation, physical interaction between SUMO and SIM is believed to be crucial. However, SUMOylation of partner proteins of the PML body such as Sp100 and p53 is not crucial (*26, 27*). We found that non-SUMO conjugated IN and PML interacted physically, but we cannot rule out the possibility that SUMOylated form of these proteins interacted with each other since SUMO-conjugated proteins are usually unstable and de-SUMOylation occurs by the actions of SUMO-specific proteinases. Thus, further study of the SUMOylation of PML and IN and its effect on their physical interaction may help clarify the meaning of SUMOylation and PML-IN interaction.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (No. 22580104) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. **Suzuki, Y., and Craigie, R.** (2007) The road to chromatin-nuclear entry of retroviruses. *Nat. Rev. Microbiol.* **5**, 187-196
2. **Iordanskiy, S., Berro, R., Altieri, M., Kashanchi, F., and Bukrinsky, M**. (2006) Intracytoplasmic maturation of the human immunodeficiency virus type 1 reverse transcription complexes determines their capacity to integrate into chromatin. *Retrovirology* **3**, 4
3. **Mitchell, R. S., Beitzel, B. F., Schroder, A. R. W., Shinn, P., Chen, H., Berry, C. C., Ecker, J. R., and Bushman, F. D.** (2004) Retroviral DNA integration-ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol.* **2**, e234
4. **Lewinski, M. K., and Bushman, F. D.** (2005) Retroviral DNA integration-Mechanism and Consequences. *Adv. Genet*. **55**, 147-181
5. **Llano, M., Vanegas, M., Fregoso, O., Saenz, D., Chung, S., Peretz, M., and Poeschla, E. M.** (2004) LEDGF/p75 determines cellular trafficking of diverse lentiviral but not murine oncoretroviral integrase proteins and is a component of functional lentiviral preintegration complexes. *J. Virol.* **78**, 9524-9537
6. **Shun, M.-C., Raghavendra, N. K., Vandegraaff, N., Daigle, J. E., Hughes, S., Kellam, P., Cherepanov, P., and Engelman, A.** (2007) LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration. *Genes. Dev.* **21**, 1767-1778

7. **Turelli, P., Doucas, V., Craig, E., Mangeat, B., Klages, N., Evans, R., Kalpana, G., and Trono, D.** (2001) Cytoplasmic recruitment of INI1 and PML on incoming HIV preintegration complexes: interference with early steps of viral replication. *Mol. Cell* **7,** 1245-1254

8. **Suzuki, Y., Yang, H., and Craigie, R.** (2004) LAP2α and BAF collaborate to organize the Moloney murine leukemia virus preintegration complex. *EMBO J.* **23,** 4670-4678

9. **Inayoshi, Y., Okino, Y., Miyake, K., Mizutani, A., Yamamoto-Kishikawa, J., Kinoshita, Y., Morimoto, Y., Imamura, K., Morshed, M., Kono, K., Itoh, T., Nishijima, K., and Iijima, S.** (2010) Transcription factor YY1 interacts with retroviral integrases and facilitates integration of Moloney murine leukemia virus cDNA into the host chromosomes. *J. Virol.* **84**, 8250-8261

10. **Studamire, B., and Goff, S. P.** (2008) Host proteins interacting with the Moloney murine leukemia virus integrase: Multiple transcriptional regulators and chromatin binding factors. *Retrovirology* **5**, 48

11. **Lallemand-Breitenbach, V., and de Thé, H.** (2010) PML nuclear bodies. *Cold Spring Harb. Perspect. Biol.* **2**, a000661

12. **Bernardi, R., and Pandolfi, P. P.** (2007) Structure, dynamics and functions of promyelocytic leukemia nuclear bodies. *Nat. Rev. Mol. Cell. Biol.* **8,** 1006-1016

13. **Everett, R. D., and Chelbi-Alix, M. K.** (2007) PML and PML nuclear bodies: implications in antiviral defence. *Biochimie* **89**, 819-830

14. **Seeler, J. S., and Dejean, A.** (2003) Nuclear and unclear functions of SUMO. *Nat. Rev. Mol. Cell. Biol.* **4**, 690-699

15. **Zamborlini, A., Coiffic, A., Beauclair, G., Delelis, O., Paris, J., Koh, Y., Magne, F., Giron, M. L., Tobaly-Tapiero, J., Deprez, E., Emiliani, S., Engelman, A., de Thé, H., and Saib, A.** (2011) Impairment of human immunodeficiency virus type-1 integrase SUMOylation correlates with an early replication defect. *J. Biol. Chem.* **286**, 21013-21022

16. **Shields, A., Witte, W. N., Rothenberg, E., and Baltimore, D.** (1978) High frequency of aberrant expression of Moloney murine leukemia virus in clonal infections. *Cell* **14**, 601-609

17. **Sato, Y., Miyake, K., Kaneoka, H., and Iijima, S.** (2006) Sumoylation of CCAAT/enhancer-binding proteinαand its functional roles in hepatocyte differentiation. *J. Biol. Chem.* **281**, 21629-21639

18. **Wang, H., Zhai, L., Xu, J., Joo, H. Y., Jackson, S., Erdjument-Bromage, T., Tempst, P., Xiong, Y., and Zhang, Y.** (2006) Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage. *Mol. Cell* **22**, 383-394

19. **Maroui, M. A., Rampin, M., and Chelbi-Alix, M. K.** (2011) Promyelocytic leukemia isoform IV confers resistance to encephalomyocarditis virus via the sequestration of 3D polymerase in nuclear bodies. *J. Virol.* **85,** 13164-13173

20. **Shen, T. H., Lin, H-K., Scaglioni, P. P., Yung, T. M., and Pandorfi P. P.** (2006) The mechanisms of PML-nuclear body formation. *Mol. Cell* **24**, 331-339

21. **Wotton, D., and Merrill, J. C.** (2007) Pc2 and SUMOylation. *Biochem. Soc. Trans.* **35**, 1401-1404

22. **Lai, L., Liu, H., Wu, X., and Kappes, J. C.** (2001) Moloney murine leukemia virus integrase protein augments viral cDNA synthesis in infected cells. *J. Virol.* **75**, 11365-11372

23. **Wu, X., Liu, H., Xiao, H., Conway, J. A., Hehl, E., Kaplana, G. V., Prasad, V., and Kappes, J. C.** (1999) Human immunodeficiency virus type 1 integrase protein promotes reverse transcription through specific interactions with the nucleoprotein reverse transcription complex. *J. Virol.* **73**, 2126-2135

24. **Yueh, A., Leung, J., Bhattacharyya, Perrone, L. A., de los Santos, K., Pu, S. Y., and Goff, S. P.** (2006) Interaction of moloney murine leukemia virus capsid with Ubc9 and PIASy mediates SUMO-1 addition required early in infection. *J. Virol.* **80,** 342-352

25. **Zhang, J. Q., Wang, J. J., Li, W. J., Huang, L., Tian, L., Xue, J. L., Chen, J. Z., and Jia, W.** (2009) Cellular protein TTRAP interacts with HIV-1 integrase to facilitate viral integration. *Biochem. Biophys. Res. Commn.* **387**, 256-260

26. **Kwek, S. S., Derry, J., Tyner, A. L., Shen, Z., and Gudkov, A. V.** (2001) Functional analysis and intracellular localizaion of p53 modified by SUMO-1. *Oncogene* **20**, 2587-2599

27. **Sternsdorf, T., Jensen, K., Reich, B., and Will, H.** (1999) The nuclear dot protein Sp100, characterization of domains necessary for dimerization, subcellular localization, and modifiction by small ubiquitin-like modifiers. *J. Biol. Chem.* **274**, 12555-12566

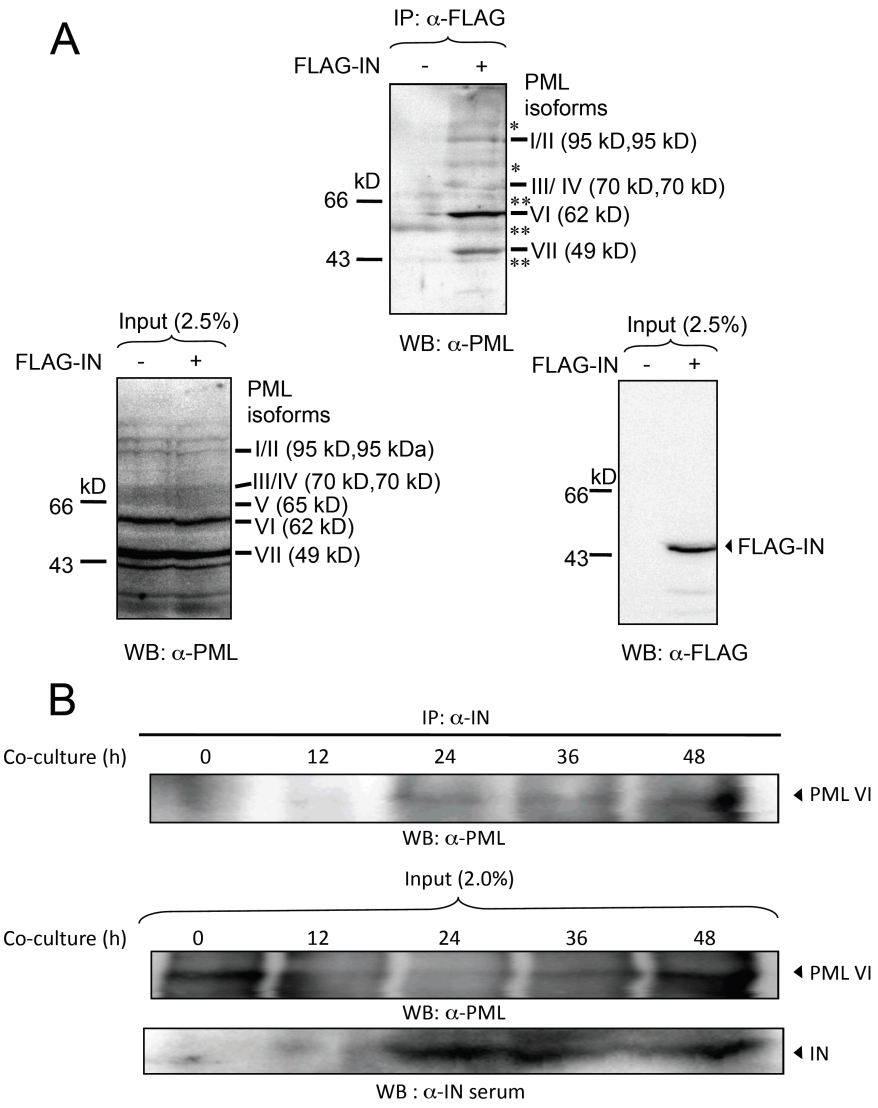

Fig. 1. PML interacts with MoMLV IN. (A) Results of coimmunoprecipitation of endogenous PML and FLAG-MoMLV IN expressed in 293FT cells (upper panel). A control without IN was included to estimate nonspecific interaction between PML and anti-FLAG antibody since the interaction between IN and some PML isoforms seemed to be weak. PML was detected with anti-PML antibody and this antibody was also used for detection of input PML isoforms (2.5%, lower left panel). FLAG-IN was detected with anti-FLAG antibody (lower right panel). PML isoforms were identified from their

molecular mass and several unidentified bands (*) were detected. The non-specific bands possibly caused by the anti-FLAG antibody were also detected (**). A result representative of several experiments is presented. (B) Results of coimmunoprecipitation of endogenous PML and IN in MoMLV infected cells. Cell extracts were prepared from cocultures of NIH3T3 and clone no. 4 cells at 0, 12, 24, 36, and 48 h. IN complex was precipitated with anti-MoMLV IN antiserum and analyzed with anti-PML antibody (upper panel). Each sample (2.0%) was used to confirm the viral IN and endogenous PML (lower panels).

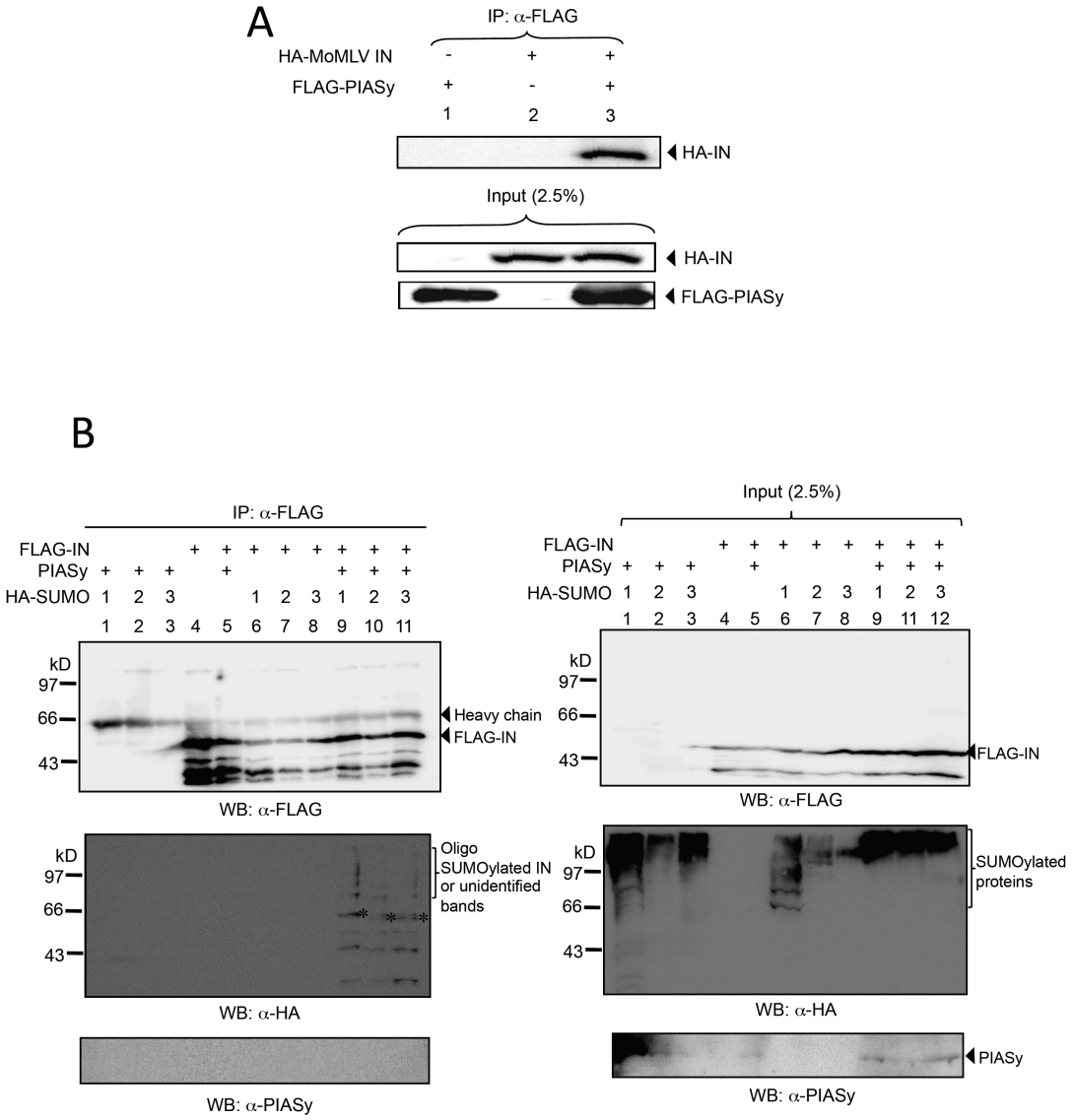

Fig. 2. PIASy is a SUMO E3 ligase of MoMLV IN. (A) Results of coimmunoprecipitation of FLAG-PIASy and HA-MoMLV IN expressed in 293FT cells (upper panel). HA-IN precipitated with anti-FLAG antibody was detected with anti-HA antibody after extensive washing with the lysis buffer. Each sample (2.5%) was used to confirm the expression of FLAG-PIASy or HA-IN (input, lower panels). A result representative of several experiments is presented. (B) PIASy stimulates SUMO

conjugation of MoMLV IN in the cells. 293FT cells were cotransfected with plasmid encoding FLAG-IN and PIASy, and HA-SUMO-1, SUMO-2, or SUMO-3. Cells were lysed in the denaturing buffer and heated at 100˚C for 5 min 48 h posttransfection followed by immunoprecipitation with anti-FLAG antibody. The SUMOylated IN proteins were analyzed with anti-FLAG antibody and anti-HA antibody (left panels). Each sample (2.5%) was used to confirm the expression of FLAG-IN, HA-SUMO-1, SUMO-2, or SUMO-3, and PIASy (input, right panels). FLAG-IN was detected with anti-FLAG antibody. HA-SUMO paralogues were detected with anti-HA antibody. PIASy was detected with anti-PIASy antibody.

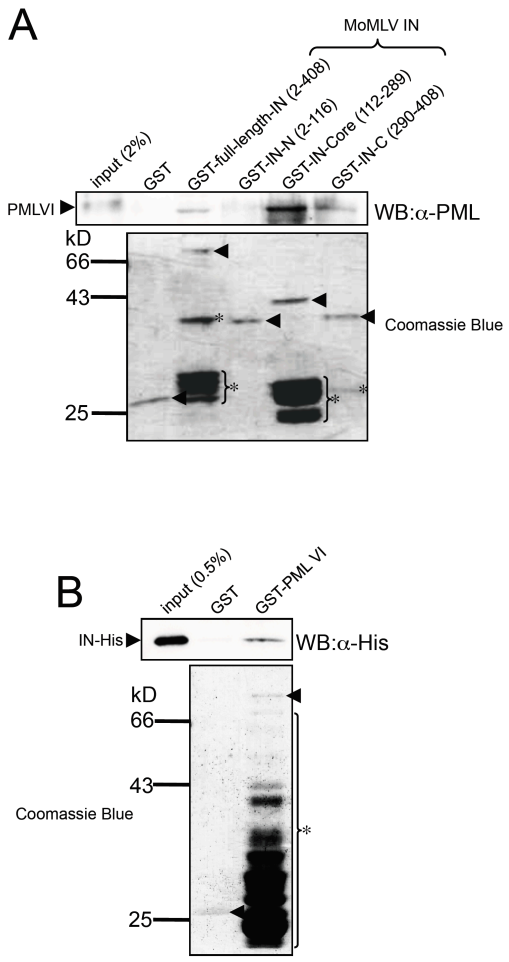

Fig 3. *In vitro* binding assay for detection of the interaction between IN and PML. (A) Results of pulldown assay of GST-tagged full-length and portions of IN with extracts of 293FT cells. PML protein was detected by Western blotting using the anti-PML antibody (upper panel). GST, GST-tagged full-length IN, and GST-tagged portions of IN were subjected to SDS-PAGE, and the gel was stained with Coomassie brilliant blue (lower panel). Arrowheads show GST-INs and GST. Asterisks indicate probable degradation products of GST-IN. A result representative of several experiments was presented. (B) Physical interaction between MoMLV IN and purified PML. GST-PML VI and purified IN-His were mixed and pulldown was performed.

GST-PML VI was confirmed with Coomassie brilliant blue. Arrowheads show GST-PML VI and GST. Asterisk indicates probable degradation products of GST-PML VI. Pulldown product was detected with anti-His antibody. A result representative of several experiments is presented.

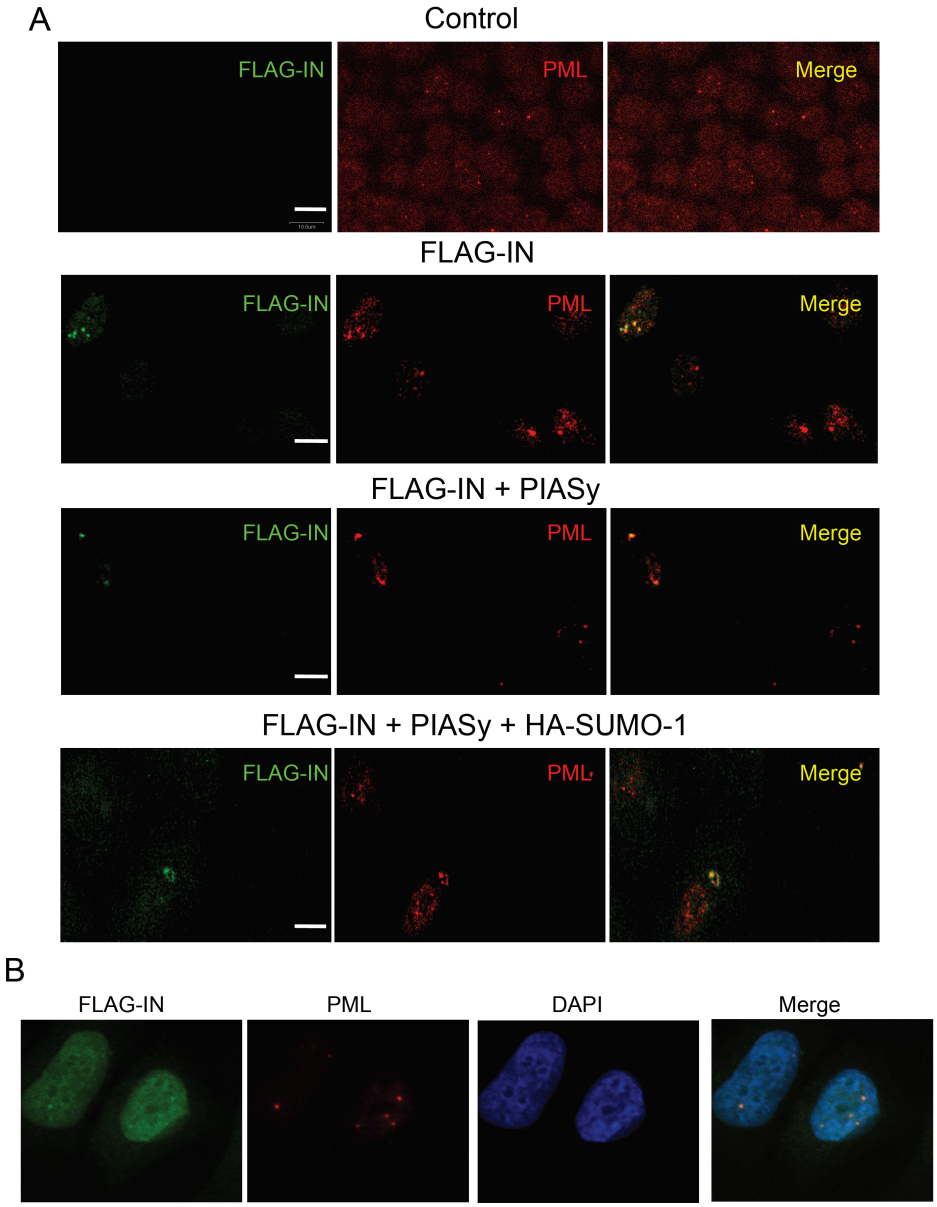

Fig 4. Colocalization of IN and PML. (A) Hela cells were transfected with expression plasmids for FLAG-IN (phCMV2/FLAG-MLV IN), PIASy and HA-SUMO-1. Pictures were taken by using laser scanning microscopy (Olympus, FV300). (B) Interaction between IN and PML in nucleus. Nuclei were stained with DAPI (Olympus, FV1000).

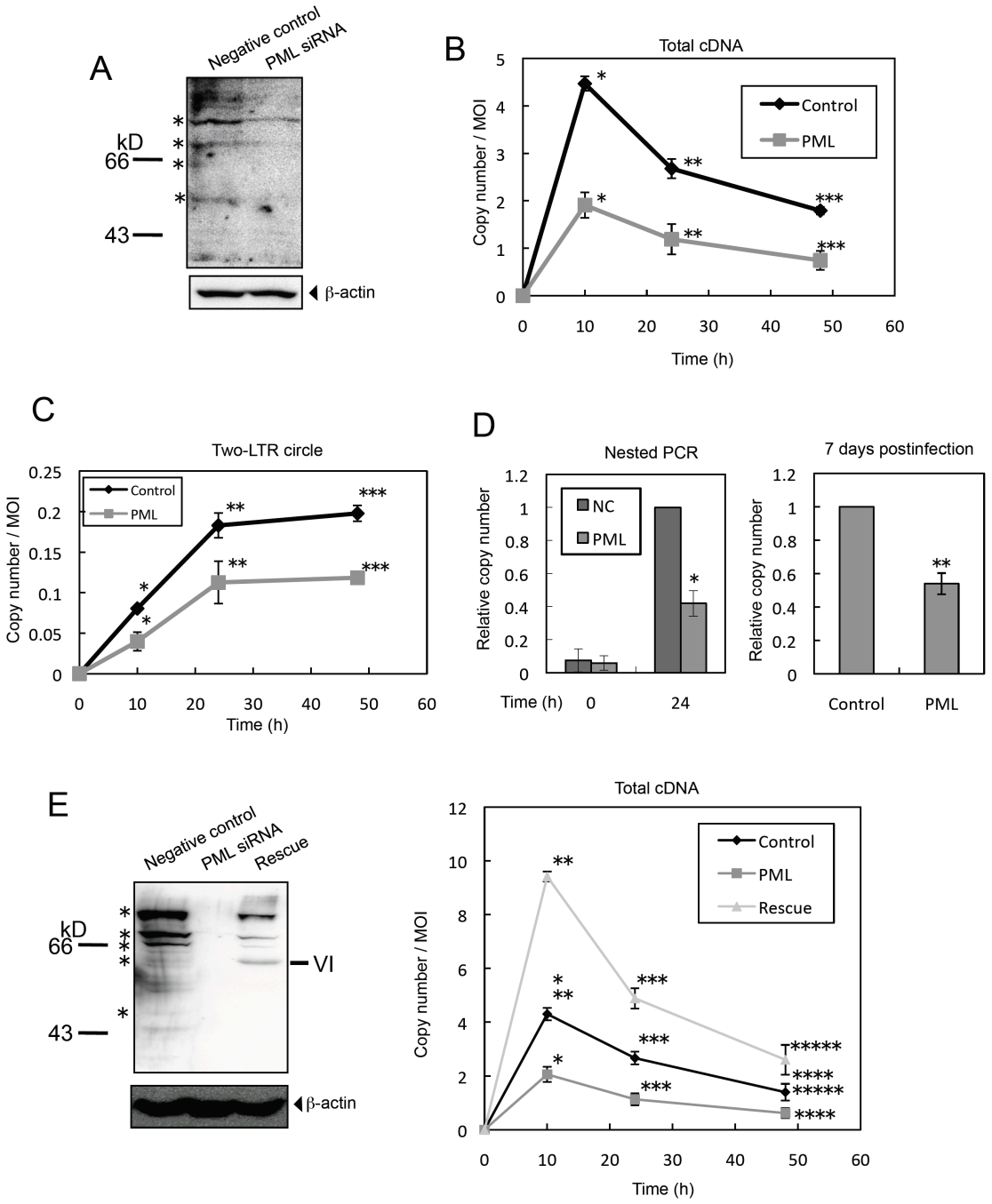

Fig 5. PML knockdown reduces total viral cDNA and integrated provirus. (A) Knockdown of PML by siRNA was confirmed by Western blotting. PML isoforms were marked with asterisks. (B and C) The amounts of total cDNA (B) and two-LTR circle cDNA (C) are shown. NIH3T3 cells were transfected twice with PML siRNA and then

infected with pQEGFP at MOI of 0.5-1.0. The values of copy number per MOI were normalized with that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Error bars represent the standard deviations of the results of three experiments. Asterisks indicate that values of PML-siRNA cells compared to those of scrambled-siRNA cells were significantly different (*, **, ***, $P < 0.001$ in panel B, *, ** $P < 0.01$, ***, $P < 0.001$ in panel C). (D) The integrated form of viral cDNA was quantified by nested PCR 24 h postinfection (left panel) and real time PCR 7 day postinfection (right panel) and normalized as described in (B) and (C). Error bars represent the standard deviations of the results of three experiments. The values corresponding to scrambled-siRNA-transfected cells were arbitrarily set to 1. Asterisks indicate that values of PML-siRNA compared to those of scrambled-RNA were significantly different (*, **, $P < 0.001$). (E) Rescue experiments by overexpression of PML VI. PML VI expression was confirmed by Western blotting. Asterisks show PML isoforms (left panel). The amounts of viral cDNA were analyzed (right panel). Asterisks indicate that values of PML-siRNA and PML-siRNA plus PML VI-expression plasmid compared to those of scrambled-RNA were significantly different (*, *****, $P < 0.05$, ***, **** $P < 0.001$, **, $P < 0.0001$). In this experiment, levels of other PML species also increased by unknown reason.

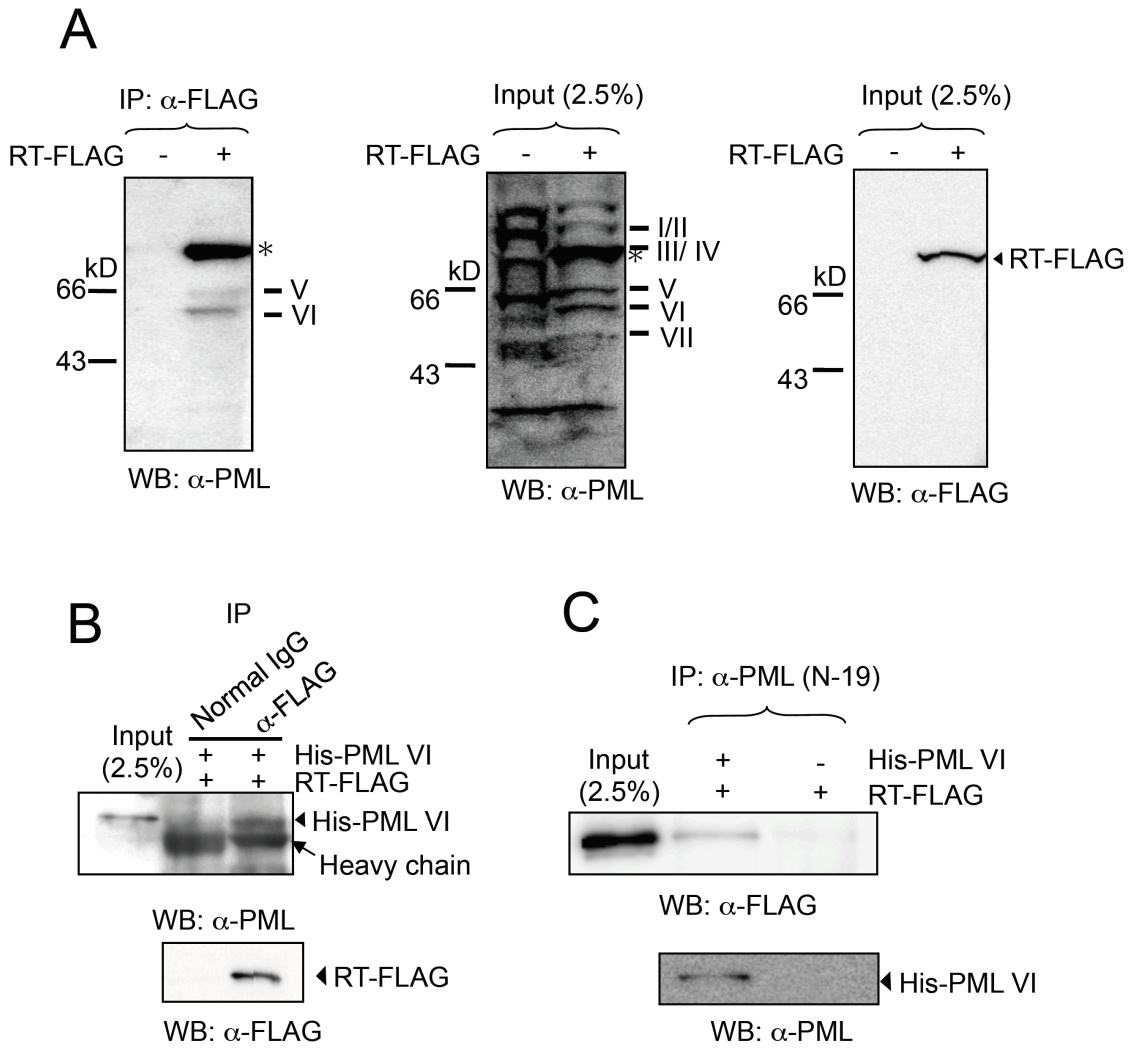

Fig. 6. PML protein interacts with MoMLV RT. (A) Result of coimmunoprecipitation of endogenous PML protein and RT-FLAG expressed in 293FT cells. RT was precipitated by FLAG M2 beads, and then PML protein was detected with the anti-PML antibody (left panel). Input (2.5%) was also analyzed with the anti-PML and -FLAG antibodies (center and right panels). Artifact band detected by the anti-PML antibody was possibly RT-FLAG since the anti-FLAG antibody detected the band that showed the same mobility (*, left and center panels). We also confirmed purified RT-FLAG reacted this anti-PML antibody (H-238) (data not shown). (B) Physical

interaction between purified MoMLV RT-FLAG and His-PML VI proteins. Upper panel: *In vitro* immunoprecipitation using purified RT-FLAG and His-PML VI, immunoprecipitation was performed with anti-FLAG antibody. Lower panel: input RT-FLAG was detected with the anti-FLAG antibody. (C) *In vitro* immunoprecipitation using purified RT-FLAG and His-PML VI. Immunoprecipitation was performed with anti-PML antibody (SantaCruz, N-19). The anti-PML antibody was confirmed not to react with RT protein. RT-FLAG was detected with the anti-FLAG antibody. A result representative of several experiments is presented.

Chapter 4
Concluding Remarks

The concluding remarks obtained each chapter are summarized as follows:

In chapter 2, we show that YY1 physically interacts with retroviral integrases and may associate with MoMLV PICs. Although MoMLV cDNA contains YY1-binding sites in the LTR sequence, mutations in these sites have no effect on the association of YY1 with PICs, suggesting that phisycal interaction between YY1 and MoMLV integrase mediated the PIC-association of YY1. In the *in vitro* studies, I also find that YY1 directly facilitate the retroviral integrase activity. Furthermore, by using the MoMLV-based retroviral vectors, I show that YY1 activates the retroviral integration although it represses the retroviral cDNA synthesis. These results indicate that YY1 has important roles in the early retroviral infection. Since YY1 is a sequence-specific transcription factor, it is reasonably assumed that YY1gives bias for the target site selection.

In chapter 3, I show that PML proteins physically interact with MoMLV integrase and reverse transcriptase, and associate with MoMLV PICs. Several PML isoform interact with these proteins, and PML VI seems to mainly bind to the viral enzymes. I also show that PIASy interacts and SUMOylates MoMLV integrase. Although SUMOylation usually affect the interaction between PML and partner proteins, SUMOylation of MoMLV integrase has no effect on the interaction. Furthermore, I

found that PML proteins activate the retroviral cDNA synthesis. These results suggest that PML proteins play important roles in the early steps of retroviral lifecycle.

I believe that these findings contribute to provide new insight into the regulation of retrovirus infection by cellular factors. However, detailed molecular mechanisms still remain unclear. Therefore, extensive studies are necessary to elucidate how they function in the retroviral lifecycle. I believe that these studies will contribute to further improvement of retroviral vectors and will stimulate further research that will help to understand the retroviral replicastion.

Publication List

Publications for this thesis

Yuuki Okino, Yujin Inayoshi, Yusuke Kojima, Shunsuke Kidani, Hidenori Kaneoka, Akiko Honkawa, Hiroshi Higuchi, Ken-ichi Nishijima, Katsuhide Miyake and Shinji Iijima

Moloney murine leukemia virus integrase and reverse transcriptase interact with PML proteins

Journal of Biochemistry (in press)

Yujin Inayoshi,* **Yuuki Okino,*** Katsuhide Miyake, Akifumi Mizutani, Junko Yamamoto-Kishikawa, Yuya Kinoshita, Yusuke Morimoto, Kazuhito Imamura, Mahboob Morshed, Ken Kono, Toshinari Itoh, Ken-ichi Nishijima, and Shinji Iijima

*Contributed equally.

Transcription Factor YY1 Interacts with Retroviral Integrases and Facilitates Moloney Murine Leukemia Virus cDNA Integration into the Host Chromosomes

Journal of Virology 84 (2010) 8250-8261

Other publicastions

Daisuke Kodama, Daisuke Nishimiya, Ken-ichi Nishijima, **Yuuki Okino**, Yujin Inayoshi, Yasuhiro Kojima, Ken-ichi Ono, Makoto Motono, Katsuhide Miyake,

Yoshinori Kawabe, Kenji Kyogoku, Takashi Yamashita, Masamichi Kamihira, and Shinji Iijima

Chicken oviduct-specific expression of transgene by a hybrid ovalbumin enhancer and the Tet expression system

Journal of Bioscience and Bioengineering doi: 10.1016/j.jbiosc.2011.10.006

Presentation at international conferences

Yuuki Okino, Yujin Inayoshi, Katsuhide Miyake, Akifumi Mizutani, Ken-ichi Nishijima, and Shinji Iijima.

Transcription factor YY1 interacts with retroviral integrases and facilitates virus cDNA integration into the host chromosomes

Japanese Association for Animal Cell Technology. P-66. Hokkaido, Japan. Sep. 2010

Acknowledgement

The works in this thesis were carried out in the Laboratory of Gene Engineering, Department of Biotechnology, Graduate school of Engineering, Nagoya University. The author would like to express his gratitude to all those who gave him the possibility to complete this thesis.

The author would like to express his grateful acknowledgement to Dr. Shinji Iijima, Professor of Graduate school of engineering, Nagoya University, for his kind guidance and encouragement throughout this study and for his review of this thesis. His wide knowledge and logical thinking have been of great value for the auther.

The author wishes to express his sincere gratitude to Dr. Hiroyuki Honda, Professor of Graduate school of engineering, Nagoya University, for his in-depth critical review, and Dr. Ken Kitajima, Professor of Graduate school of Bioagricultural Sciences, Nagoya University, for his kind advices and suggestions. The author is deeply grateful to Dr. Katushide Miyake, Associate professor of Graduate school of engineering, Nagoya University, for his detailed and constructive comments, and for his important support throughout this work. The author tenders his gratitude to Dr. Ken-ichi Nishijima, Assistant professor of Graduate school of engineering, Nagoya University, for his valuable comments.

The author would like to give a special thanks to all members of Laboratory of Gene Engineering for friendship and helpful discussion.

Finally, the author is heartily gratitude to his birth parents, Akihiro and Kiyoka Narimatsu, and adapted parents, Kunikiyo and Takeko Okino, for their loving support. Without their encouragement and understanding, it would have been impossible to finish this work.

Nagoya, Japan, January 2012.

Yuuki Okino