CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and equipments

Chemicals, kits and instruments used in this study are shown in **Appendix A**. Cell lines and microorganisms are demonstrated in **Appendix B**. All of antibodies and conjugated antibodies were listed in **Appendix C.** List of restriction enzymes are detailed in **Appendix D**. The recipes for reagent preparations are shown in **Appendix E.**

2.2 Zinc Finger Protein Design

Studying order to identify target sites within the DNA sequence, the 36 bp HIV-1 DNA sequence covered the area of 2-LTR-circle junctions (5' TGG AAA ATC TCT AGC AGT ACT GGA TGG GCT AAT TCA 3') (Whitcomb et al., 1990) was submitted to the ZF Tools server of the Barbas Laboratory of the Scripps Research Institute (Mandell and Barbas, 2006). The minimum target size of 18 base pairs was set to obtain the output DNA target sequences and amino acid sequences for the six fingers of the ZFP using "Search DNA Sequence for Contiguous Target Sites" mode and by searching both forward and reverse strand. This amino acid sequence was then reverse-translated into a nucleotide sequence. Codon usage of the amino acid sequences of the selected ZFP was optimized by using the JAVA Codon Adaptation Tool (JCat) (Grote et al., 2005) and by manual optimization. The designed

ZFP was further modified with flanking *Xcm*I and *Sma*I restriction endonuclease sites. The full-length optimized DNA sequence was sent for full-gene synthesis to Blue Heron Biotechnology (Bothell, WA). Predicted properties of the resulting proteins were computed using ExPaSy proteomics tools (Wilkins et al., 1999). Prediction of protein subcellular localization was done using the LOCtree program (Nair and Rost, 2005).

2.3 Plasmid construction

The full-length optimized DNA sequence of 2LTRZFP was synthesized and cloned into pUC19 vectors by Blue Heron Biotechnology. The 2LTRZFP gene fragment was ligated to pTriEx-4-GFP using the flanking *Xcm*I and *Sma*I sites to construct pTriEx-4-2LTRZFP-GFP, creating an N-terminal His6 - fusion protein with GFP in the C-terminal part. This plasmid has a multiple expression system driven by the CMV, T7, or p10 promoters. The ligation product was transformed into the competent *E. coli* XL-1 Blue cells and plated on Luria-Bertani (LB) agar containing 100 μg/ml of ampicillin. The plasmid miniprep was performed using a QIAGEN Miniprep Kit (Qiagen, Hilden, Germany). The constructed plasmid was preliminary identified by the restriction endonucleases *Xcm*I and *Sma*I. PCR and DNA sequencing were performed for confirmation.

2.4 Expression and purification of His6-2LTRZFP-GFP

E. coli Origami B (DE3) was used as the expression strain. A 10 ml Terrific broth preculture, supplemented with 100 ug/ml ampicillin, 12.5 ug/ml kanamycin, and

12.5 ug/ml tetracycline was incubated at 37 ºC until reaching an absorbance at 600 nm of about 1.0 - 1.8. The preculture was diluted 1:100 into 100 ml Terrific broth medium with 100 µg/ml ampicillin, 12.5 ug/ml kanamycin, 12.5 ug/ml tetracycline and supplemented with 100 µM Zn_2SO_4. The culture was incubated at 37 ºC. When an absorbance about 1.0 at 600 nm was reached, the bacterial culture was induced by 0.1 mM isopropyl β-*D*-thiogalactopyranoside (IPTG) at 30 ºC for overnight. Bacteria were then harvested by centrifugation (7,500 *g* at 4 ºC for 15 min). The bacterial pellets were resuspended in 6.25 ml of *B-PER II* Bacterial Protein Extraction Reagent (Pierce, Rockford, IL, USA) and lysed by ultrasonication in an ice bath. The lysate was centrifuged at 15,000 *g* for 20 min at 4 ºC. The supernatant was collected and filtered with microfiltration membranes (0.22 µm pore size). The clear solution containing His6-2LTRZFP-GFP was applied to His-bind column chromatography (Novagen, San Diego, CA) for protein purification. The eluated fraction containing His6-2LTRZFP-GFP was observed using Coomassie brilliant blue R250 stain (Bio-Rad, Hercules, CA). The purified protein was kept in final concentration 25% (*W*/*V*) glycerol for long term storage at −80 ºC. The protein concentration was quantified by using the Micro-BCA protein assay (Pierce). All the samples were analyzed by SDS-PAGE. Western blot analysis was performed on a Hybond-P polyvinylidene fluoride (PVDF) membrane. (Amersham Bioscience, Piscataway, NJ). After being blocked with 5% skim milk in PBS, the proteins were probed with anti-his-tag monoclonal antibody (Genscript, Piscataway, NJ) as a primary antibody and horseradish peroxidase (HRP) - labeled goat anti-mouse immunoglobulins (Sigma, St Louis, MO) as a secondary antibody, using the ECL system (GE Healthcare, Buckinghamshire, UK).

2.5 Cell culture and transfection of HeLa cells

HeLa cervical carcinoma cells were kindly obtained from Dr. A. Lieber, University of Washington, Seattle, WA. HeLa cells ($1x10^5$) were seeded onto 24 well-plate in humidified atmosphere under 5% CO_2 at 37 °C in Dulbecco's Modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) containing penicillin (100 units/ml), streptomycin (100 ug/ml) and 2 mM L-glutamine, supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) for 24 h before transfection. Transfection mixture was prepared by adding 1 ug of the pTriEx-4-2LTRZFP-GFP or control vector pTriEx-4-GFP and 2 ul of GeneJamer (Stratagene, La Jolla, CA) into DMEM up to 200 ul. Then, the mixture was incubated at room temperature for 10 min. The culture supernatant was discarded and 300 ul of fresh DMEM containing 10% FBS and antibiotics were added. The transfection mixture was added to the cells and incubated at 37 °C in 5% CO_2 for 5 h. 500 ul of DMEM containing 10% FBS and antibiotics were added and further cultured at 37 °C in 5% CO_2 for 24-48 h. Green fluorescent protein (GFP) was observed under a fluorescent microscope.

2.6 Double-stranded DNA preparation

Double-stranded DNA (ds-DNA) of specific and non-specific target DNA was prepared for testing the binding activity of 2LTRZFP-GFP. A pair of specific ds-DNA (sense) was designed as follows: 5'-AAA TCT *CTA GCA GTA CTG GAT GGG* CTA ATT-3' and a pair of non-specific ds-DNA (sense) was also designed as follows 5'-TGA CAG TGC TAG CGT ATC ATC TAG TCG ACG -3'. The specific sequences to 2LTRZFP-GFP are shown in italic. The reaction mixture (100 ul) for annealing was

composed of each 600 pmol single-stranded DNA (ss-DNA) and complementary strand in 50 mM NaCl. The mixture was heated at 95 °C for 5 min and then slowly cooled to room temperature for 90 min. For immobilization of ds-DNA on a sensor chip SA (Biacore AB, Uppsala, Sweden) for surface plasmon resonance (SPR), the 3' end of the anti-sense strand of specific target DNA were labeled with biotin.

2.7 Surface plasmon resonance (SPR)

SPR was carried out on a BIACORE 2000™ biosensor (Biacore AB). The target DNA duplexes were immobilized by injecting the biotinilated specific ds-DNA of 0.5 ug/ml in 0.3 M NaCl on a Sensor Chip SA (Biacore AB) at a flow rate 5 ul/min in running buffer (HBS-EP), containing 10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, 0.005% [v/v] surfactant P20 (Biacore AB). Typically 800-1000 RUs of the target ds-DNA were immobilized. After DNA immobilization, the chip was washed with 50 mM NaOH/1M NaCl and then primed with zinc buffer [10 mM Tris-HCl pH 7.5, 90 mM KCl, 1 mM $MgCl_2$, 90 µM $ZnSO_4$, 5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonylfluoride (PMSF)] which was used as a running buffer for a period of binding analysis. ZFP was diluted in zinc buffer and 60 ul were injected at a flow rate 20 ul/min, followed by a dissociation phase of 180 s. Before each injection, the base-line stability was achieved by injecting 1M NaCl for 2 min. To calculate the binding affinity (K_D), the kinetic parameters were evaluated with BIA evaluation software 3.1 (Biacore AB) using a 1:1 binding model with mass transfer. For competitive SPR, 2LTRZFP-GFP was incubated with different concentrations of non-biotinylated ds-DNA of its target ds-DNA, and non-specific ds-DNA in zinc buffer for 15 min before injection.

2.8 Electrophoretic mobility shift assay (EMSA)

2LTRZFP-GFP or GFP were incubated with 250 nM of DNA duplex at room temperature for 1 h in zinc buffer. Total volumes were 10 ul per reaction. The reaction mixtures were mixed with 2ul of 6X EMSA gel-loading solution (component D) of EMSA kit [E33075] (Invitrogen, Paisley, UK) before being loaded on 5% non-denaturing polyacrylamide gels using 100V, 30 min. Gels were stained by using two fluorescent dyes for detection- SYBR® green EMSA nucleic acid gel stain (component A) and SYPRO® ruby EMSA protein gel stain (component B) by following the protocol from the same kit. The stained gels were imaged at an excited state of 488 nm by using a Typhoon Trio phosphorImager (GE Healthcare Biosciences, Piscataway, NJ).

2.9 Construction of expression vectors

The 2LTRZFP-GFP and Aart-GFP gene fragments were amplified from pTriEx-4-2LTRZFP-GFP, which has been previously described above (Sakkhachornphop *et al.*, 2009), and pTriEx-4-Aart-GFP, respectively. Aart is a 6-zinc finger protein designed to recognise a unique 18-bp target site not found in the human genome, and this gene was used as a control (Dreier *et al.*, 2001). Both genes were cloned into pRRLSIN.cPPT.mPGK-GFP.WPRE using the flanking *Xba*I and *Sal*I sites, which led to the construction of pRRLSIN.cPPT.mPGK.2LTRZFP-GFP.WPRE and pRRLSIN.cPPT.mPGK.Aart-GFP.WPRE, and these fusion genes encoded N-terminal His6 and C-terminal GFP. These plasmids were driven by the murine phosphoglycerate kinase (mPGK) promoter. The ligation product was electrotransformed into competent *Escherichia coli* XL-1 Blue cells and plated on

Luria-Bertani (LB) agar containing 100 μg/ml ampicillin. The plasmid minipreps were performed using the PureLink Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA). The sequences of the constructed plasmids were preliminary confirmed by restriction digest with *Xba*I and *Sal*I. PCR and DNA sequencing were performed for further confirmation of the sequences. The p156LLSIN.cPPT.mPGK.RFP.WPRE construct was then prepared for the pseudotyped lentiviral challenge study. The RFP gene fragment was amplified from pDsRed2-N1 (Clontech, Palo Alto, CA), and this gene was cloned in an analogous fashion to 2LTRZFP-GFP and Aart-GFP. The HIV-1 molecular clone used in this study was pNL_{4-3}, which was obtained from the AIDS Research and Reference Reagent Program.

The 2LTRZFP-GFP and Aart-GFP gene fragments were also cloned into the pCEP4 vector (Invitrogen, La Jolla, CA) using the flanking *Nhe*I and *Not*I sites to construct pCEP4-2LTRZFP-GFP or pCEP4-Aart-GFP, respectively. Expression was driven by the cytomegalovirus (CMV) immediate-early enhancer/promoter. The Epstein-Barr virus replication origin (oriP) and nuclear antigen (encoded by the EBNA-1 gene) were carried by this plasmid to permit extrachromosomal replication in mammalian cells. The ligation product was electrotransformed into competent *E. coli* XL-1 Blue cells and plated on Luria-Bertani (LB) agar containing 100 μg/ml ampicillin. Plasmid minipreps were performed using the PureLink Quick Plasmid Miniprep Kit. The plasmid sequences were preliminarily confirmed by digestion with *Nhe*I and *Not*I. PCR and DNA sequencing were performed for further sequence confirmation.

2.10 Cell cultures

The packaging cell line, 293T, was maintained in Dulbecco's Modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) containing penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine and 10% foetal bovine serum (FBS) (HyClone, Cramlington, UK). The CD4-positive human T-lymphocytic cell lines (SupT1 and stable lines susceptible to HIV-1 infection) were grown in RPMI-1640 medium supplemented with 10% FBS in a humidified atmosphere with 5% CO_2 at 37 °C.

2.11 Production of VSV-G pseudotyped lentiviral particles

The third-generation pseudotyped lentiviral particles containing the vesicular stomatitis virus G glycoprotein (VSV-G) were produced in 293T cells transiently cotransfected with four vectors. 293T cells (3.5×10^6 cells) were seeded into 10-cm dishes, and they were transfected using Lipofectamine and Plus Reagent (Invitrogen, Carlsbad, CA). The following amounts of DNA were used for the transfection experiment: 2.22 μg of pRRLSIN.cPPT.mPGK.2LTRZFP-GFP.WPRE, pRRLSIN.cPPT.mPGK.Aart-GFP.WPRE, or pRRLSIN.cPPT.mPGK-RFP.WPRE; 1.44 μg of the conditional packaging construct, pMDLgag/polRRE (for expression of *gag* and *pol* genes); 0.55 μg of the pRSV-Rev construct (for expression of *rev* cDNA); and 0.77 μg of the pMD.G construct (which encodes a heterologous envelope for VSV-G). After 5 hr, the transfection mixture was replaced with growth medium, and the cells were allowed to grow for 48 or 72 hr. The viruses were harvested from the culture supernatant, and they were filtered through a Millipore Millex-HA 0.45 μm filter unit, aliquoted, and frozen at -80 °C. Viral culture supernatants were lysed

with 0.2% Triton X-100, and a p24 Ag ELISA was performed using the Genetic Systems HIV-1 Ag EIA Kit (Bio-Rad Laboratories, Redmond, WA). Viral load was determined using the COBAS AMPLICOR HIV-1 Monitor Test (v1.5; Roche Molecular Systems, Inc., Branchburg, NJ).

2.12 HIV-1 viral stocks

293T (3.5×10^6 cells) were plated in 10-cm dishes. After approximately 12 hr, the cells were transfected with 5 μg of the pNL_{4-3} plasmid using Lipofectamine and Plus Reagent. After 5 hr, the transfection mixture was replaced with 10 ml of growth medium, and the cells were allowed to grow for 48 or 72 hr. The viruses were harvested from the culture supernatants, and they were filtered through a Millipore Millex-HA filter unit 0.45 μm, aliquoted, and frozen at -80 °C. The p24 Ag ELISA was performed using the Genetic Systems HIV-1 Ag EIA Kit. The viral load was determined using the COBAS AMPLICOR HIV-1 Monitor Test (v1.5).

2.13 Determination of viral production by p24 Ag ELISA

The concentration of p24 Ag in culture supernatant was measured by using the Genetic Systems HIV-1 Ag EIA kit. Firstly, 50 μl of Sample Diluent was applied in to a sample wells. Samples were prepared mixed 150 μl of the culture supernatants with 15 μl of 10% Triton X-100. Then 150 μl of prepared samples were added directly into the wells and the plate was then incubated for 60 min at 37 ˚C dry-heat incubator. Reaction wells were washed 5 times with Washing Solution provided in the kit and subsequently added 100 μl of Working Conjugate Solution 1 (biotinylated

sheep anti-p24 antibody, incubated plate for 30 min at 37 ˚C dry-heat incubator. Then, 100 µl of Working Conjugate Solution 2 (avidin-HRP) was added into wells after washing step 5 times and incubated for 30 min at 37 ˚C. Reaction wells were washed 5 times and subsequently added 100 µl of TMB Substrate Solution and allowed reaction to develop in the dark for 5-30 min at room temperature. Reactions were stopped by adding 100 µl of Stopping Solution and read absorbance on a spectrophotometer at 450 nm.

2.14 Generation of stable lines expressing either 2LTRZFP-GFP or Aart-GFP by lentiviral gene transfer

293T (1×10^4 cells) and SupT1 (1×10^5 cells) cells were seeded in 24-well plates and incubated with 1 ml of VSV-G pseudotyped lentiviral particles at 20 multiplicity of infection (MOI) in growth media containing polybrene (8 µg/ml). Spinoculation was performed by spinning the plates at 800 *g* at room temperature for 1.5 hr. The cells were then incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 24 hr. After 24 hr, the culture supernatant was replaced with fresh growth medium, and the cells were maintained for 3 days. The efficiency of the stable transduction was determined based on observations using fluorescent microscopy and flow cytometry. A mixed population of transduced SupT1 cells was isolated by limiting dilution for single clone selection over the course of 3 weeks.

2.15 Generation of SupT1 cells stably expressing either 2LTRZFP-GFP or Aart-GFP by non-viral gene transfer

SupT1 (1×10^6 cells) were electrotransfected with 5 μg of either pCEP4-2LTRZFP-GFP or pCEP4-Aart-GFP using the Nucleofector transfection reagent V (Amaxa, Koeln, Germany) following the manufacturer's protocol for T-16 cells. The cells were maintained for 3 days. The efficiency of transfection was determined by fluorescent microscopy and flow cytometry. SupT1 cells that stably expressed the desired genes were continuously selected by limiting dilution in 500 to 1000 μg/ml hygromycin B. The clones were maintained in 200 μg/ml hygromycin B for long-term culture.

2.16 Quantitation of integrated HIV-1 DNA (provirus) by an *Alu-gag* qPCR assay

Two-step PCR amplification was performed according to the previously described protocol (O'Doherty et al., 2002; Agosto et al., 2007) with some modifications. Briefly, the first-round PCR was performed on extracted DNA from SupT1 and/or SupT1 stable cell lines expressing 2LTRZFP-GFP or Aart-GFP using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). The following primer sequences were used to detect HIV-1 integration: *Alu* forward, 5'-GCC TCC CAA AGT GCT GGG ATT ACA G-3'(O'Doherty *et al.*, 2002); and HIV Gag reverse, 5'- GTT CCT GCT ATG TCA CTT CC -3' (Agosto *et al.*, 2007). A Gag primer (GagREV_B, 5'- CGT TCT AGC TCC CTG CTT GCC CAT AC-3') was designed to detect the background level of integrated transgenes resulting from lentiviral gene transfer. The reactions were performed in a volume of 25 μl containing

2.5× master mix (5 Prime, Gaithersburg, MD), 400 nM *Alu* forward primer, and 400 nM HIV Gag reverse or GagREV_B primer. The thermal cycler (MJ MiniThermal Cycler and MiniOpticon Real-Time PCR System, Bio-Rad) was programmed to perform a 2-min hot start at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing at 62 °C for 15 sec, and extension at 72 °C for 3.5 min.

The second-round RU5 kinetic PCR was performed using 10 µl of diluted (1:8) first-round amplicon. The primer sequences were as follows: R_FWD, 5'-TTA AGC CTC AAT AAA GCT TGC C-3'; and U5 _REV, 5'-GTT CGG GCG CCA CTG CTA GA-3' (Liszewski *et al.*, 2009). The RU5 molecular beacon probe, which was labelled at its 5'-terminus with the 6-carboxyfluorescein (FAM) reporter and at its 3'-terminus with the BlackBerry Quencher (BBQ), had the following sequence: 5'-FAM-CCA GAG TCA CAC AAC AGA CGG GCA CA–BBQ-3' (Liszewski *et al.*, 2009). The reactions were performed in a final volume of 25 µl containing 2× DyNAmo probe qPCR master mix (Finnzymes, Espoo, Finland), 400 nM RU5 (R_FWD) primer, 400 nM RU5 (U5 _REV) primer, and 140 nM RU5 molecular beacon probe. The reactions were performed on a MJ Mini Thermal Cycler and MiniOpticon Real-Time PCR System with the following program: 20-sec hot start at 95 °C followed by 50 cycles of denaturation at 95 °C for 3 sec and annealing and extension at 63 °C for 30 sec.

A primer-probe set designed to quantify the copy number of the cellular gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), was used to adjust the amount of DNA in each qPCR assay. The GAPDH primer sequences were as follows:

GAPDH_FWD, 5'-GAA GGT GAA GGT CGG AGT C-3'; and GAPDH_REV, 5'-GAA GAT GGT GAT GGG ATT TC-3'. The GAPDHTM molecular beacon probe was labelled at its 5'-terminus with a FAM reporter and at its 3'-terminus with BBQ. The GAPDHTM sequence was as follows: 5'-FAM-CAA GCT TCC CGT TCT CAG CCT-BBQ-3'. The reactions were performed in a final volume of 25 µl containing 2× DyNAmo probe qPCR master mix, 320 nM GAPDH_FWD primer, 320 nM GAPDH_REV primer, and 240 nM GAPDHTM molecular beacon probe. The reactions were performed on an MJ Mini Thermal Cycler and MiniOpticon Real-Time PCR System with the following program: 10-min hot start at 95 °C followed by 45 cycles of denaturation at 95 °C for 30 sec and annealing and extension at 63 °C for 1 min.

2.17 Challenge of 2LTRZFP-GFP-expresing cells with VSV-G pseudotyped lentiviral RFP

Two hundred thousand 293T cells or stably transduced 293T cells (2LTRZFP-GFP and Aart-GFP) were seeded into 6-well plates and then incubated overnight with diluted VSV-G pseudotyped lentiviral RFP viral supernatant at 1 or 10 MOI in 3 ml of growth medium. The culture supernatant was then replaced with growth medium. Every 2 days after the challenge, the cells were subcultured at a 1:6 dilution. Flow cytometry was performed weekly by gating on green and red channels. The expression of RFP was analysed in GFP-positive cells by gating 100,000 cells. For confocal imaging, the cells were seeded on 20-mm coverslips in a 6-well plate. The next day, the cover slips were washed with PBS and fixed in 4% formaldehyde for 15 min at room temperature followed by permeabilisation with 0.1 % Triton X-100 in

PBS for 5 min. The cells were stained with DAPI, and the slides were mounted with Vectashield before microscopy analysis. The samples were viewed at 100× magnification using a Carl Zeiss MicroImaging LSM 700 confocal laser scanning microscope (Germany), and the data were analysed using ZEN 2010 software.

2.18 Flow cytometric analysis for CD4 expression

SupT1 cells and SupT1 cells stably expressing either 2LTRZFP-GFP or Aart-GFP were collected and washed three times with PBS. The Fc receptor on the cells (1×10^5 cells) was blocked by incubation with human AB serum on ice for 30 min. To 50 µl of blocked cells, 50 µl of 20 µg/ml purified anti-CD4 mAb (MT4-3; kindly provided by Prof. Dr. Watchara Kasinrerk) in 1% BSA-PBS-NaN_3 was added, and the samples were incubated on ice for 30 min. The cells were then washed twice with 1% BSA-PBS-NaN_3 and resuspended in 20 µl of 1% BSA-PBS-NaN_3. Subsequently, 25 µl of polyclonal Rabbit Anti-mouse Immunoglobulins/RPE, Rabbit $F(ab')_2$ (DAKO, Denmark) was added to the samples, and the samples were incubated on ice for 30 min. Finally, the cells were washed three times with 1% BSA-PBS-NaN_3 and fixed with 1% paraformaldehyde in PBS. The fluorescent reactivity of the stained cells was analysed by flow cytometry.

2.19 HIV-1 infection

SupT1 cells stably expressing either 2LTRZFP-GFP or Aart-GFP were maintained in growth medium for at least 4 weeks before HIV-1 infection. The cells were infected with cell-free HIV-1_{NL4-3} at 0, 1, and 5 MOI for 8 and 16 hr. The cells were then washed three times with prewarmed, serum-free medium and suspended in growth medium. Every 2 days, the cells were split (1:2) to maintain a cell density of approximately 1×10^6 cells/ml, and the culture supernatants were collected for the HIV-1 p24 antigen assay (Genetic Systems HIV-1 Ag EIA Kit, Bio-Rad). The cell pellets were kept at -80 °C until the determination of the inhibition of intracellular HIV-1 integration by *Alu-gag* qPCR. Cell viability was monitored by trypan blue exclusion staining.