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Original article

Role of Nup98 in nuclear entry of human immunodeficiency virus type 1 cDNA

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Abstract

Human immunodeficiency virus type 1 (HIV-1), like other lentiviruses, can infect non-dividing cells. The lentiviruses are most likely to have evolved a nuclear import strategy to import HIV-1 cDNA and viral protein complex through the nuclear pore complex (NPC) formed by nucleoporin proteins (Nup). In this study, we found that synthesis of integrated and 2LTR but not full-length form of HIV-1 cDNA was clearly impaired in culture via transduction of vesicular stomatitis virus matrix protein (VSV M), an inhibitor protein, through binding to the phenylalanine-glycine (FG) repeat region of Nup98. The impairment of synthesis of integrated and 2LTR DNA with VSV M was restored by ectopic overexpression of Nup98. A series of experiments using Nup98-depleted NPC by the small interfering RNA (siRNA) technique showed specific impairment of NPC structure and some functions, including nuclear import of HIV-1 cDNA. Our results suggest that Nup98 on the NPC specifically participates in the nuclear entry of HIV-1 cDNA following HIV-1 entry.

Keywords: Nucleoporin; NPC; HIV-1; Nuclear import

1. Introduction

The Retroviridae family of viruses can reverse transcribe their RNA genome into cDNA and then integrate the cDNA into host chromosomes. The lentiviruses (e.g. HIV) are distinguished by their ability to infect non-dividing cells, whereas the gamma-retroviruses (e.g. Moloney murine leukemia virus) require nuclear membrane dissolution to access the host cell DNA [1]. Thus, the lentiviruses are most likely to have evolved a nuclear import strategy, which allows their cDNA to cross the nuclear membrane independently of mitosis. In the case of human immunodeficiency virus type 1 (HIV-1), mitosis-independent replication was initially shown in terminally differentiated macrophages in vitro [1–3]. The mitosis-independent replication of HIV has also enabled the generation of integration-competent gene transfer vectors with promising therapeutic applications in a variety of non-dividing cellular hosts, including neurons [4], myocytes [5], and retinal cells [6]. To facilitate integration into a host DNA, a preintegration complex (PIC) is generated in the cytoplasm immediately after completion of reverse transcription. The PIC can be isolated successfully from in vitro freshly HIV-1-infected or HIV vector-infected cells and was recently shown to have the ability to traverse the nuclear pore complex (NPC) [1,7]. The NPCs serve as the conduits for bi-directional transport of macromolecules. Translocation across the NPC into the nucleus and from the nucleus into the cytoplasm is governed by a class of proteins known as importins and exportins (transport receptors), respectively. Both are members of the karyopherin family. The transport receptors engage the appropriate import or export signals and mediate their transport [8,9]. The PIC contains a double-strand linear cDNA as well as at least four viral proteins: matrix (MA), reverse transcriptase (RT), integrase (IN), and viral protein R (VPR), and has a diameter of approximately 56 nm, which greatly exceeds the 25 nm central channel of the NPC [1,7,10]. The NPC has a large supramolecular structure formed of ~50 unique proteins in eukaryotic cells, termed nucleoporins (Nup) [8,9,11,12]. High-resolution electron microscopic images of NPCs reveal an eightfold

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symmetric structure, formed by nuclear and cytoplasmic rings and central spoke complex. The Nups often contain multiple phenylalanine-glycine (FG) dipeptide repeats clustered in domains, which in vertebrates are glycosylated by addition of *O*-linked *N*-acetylglucosamine (GlcNAc). Some of these Nups are localized asymmetrically at the NPC [9,11]. The asymmetric distribution of nucleoporins and the different affinities for import and export complexes may be important in determining the direction of transport [13,14]. Recent studies reported that importin 7 is involved in the nuclear entry of HIV-1 PIC as one of the main transport receptors [15]. However, the steps involved in the NPC remain largely undefined. In the present study, we show that nuclear import of HIV-1 cDNA requires NPC, and Nup98 has a role in nuclear entry of HIV-1 cDNA.

2. Materials and methods

2.1. Chemical treatment

Aphidicolin (APH) (Sigma Chemical Co., St. Louis, MO, USA), actinomycin D (ActD) (Sigma), zidovudine (AZT) (Sigma) or leptomycine B (LMB) (Sigma) was used. APH treatment (5 μg/ml) started 24 h before HIV-1 vector infection. AZT treatment started at the time of infection. LMB was added 2 h after infection. ActD was added 5 h after infection. DNA was extracted 24 h after infection.

2.2. Transfection

Human 293T cells were maintained in D-MEM containing 10% fetal calf serum (FCS). 293T cells were transfected with vesicular stomatitis virus matrix protein (VSV M)-, Nup98- or small interfering RNA (siRNA)-expressing DNA using calcium phosphate methods.

2.3. Quantitative polymerase chain reaction (PCR) assay

For the detection and quantification of individual forms of HIV-1 DNA, full-length/1LTR circle, 2LTR circle and integrated forms, we used a set of primer pairs and fluorogenic probes, as described previously [16,17]. PCR was performed using an ABI PRISM 7700 sequence detection system (PE-Applied Biosystems, Foster City, CA, USA) and TaqMan Universal PCR Master Mix (PE-Applied Biosystems). Cycling conditions included a hot start (50 °C for 2 min, 95 °C for 10 min), followed by 40 cycles of denaturation (95 °C for 15 s) and extension (60 °C for 1 min). To measure the integrated DNA, an *Alu*-sequence-specific sense primer and an antisense HIV-specific primer were used in the first PCR and subsequently 1000-fold diluted products were subjected to real-time PCR assay for measurement of R/U5 DNA, as described previously [16,17].

2.4. Cell-cycle analysis

Cell-cycle progression was examined by single-color flow cytometric analysis of the DNA content stained with 50 μg/ml of propidium iodine (Sigma).

2.5. DNA constructs and recombinant protein expression

Small interference RNA (siRNA)-expressing plasmid DNAs were constructed using the method described by Miyagishi and Taira [18]. The sequences inserted in the *Bfu*AI site of pU6i cassette, immediately downstream of the U6 promoter, were as follows: Nup98-targeted siRNA (siN98), 5′-CACCG**AATATGAAAG***T***AAGT***T***A***T***T**ATAGAATTACATCAAGGGAGA**TTAGTGACTTGCTTTCATATTC**-TTTTTATGC-3′; firefly luciferase-targeted siRNA (siLuc), 5′-CACC**GTGCG***T***TG***T***TGGTG***T***TAA***T***CC**ATCTCCCTTGATGTAATTCTA**GGGTTGGCACCAGCAGCGCAC**-TTTTTATGC-3′. Bold-lettered nucleotides are the siRNA sequences, italic nucleotides are mutated, and underlined nucleotides are loop sequences. The siRNA-expressing DNA fragment was also inserted into the *Eco*RI site of a lentivirus vector DNA, pCS-CDF-EH2K^k, and an enhanced green fluorescence protein (EGFP) fragment between the *Age*I and *Xho*I sites of pCS-CDF-EG-PRE [19] was replaced with a H-2K^k fragment (Daiichi pure chemicals, Tokyo, Japan).

HA-tagged human Nup98-expressing plasmid DNA (p37R-HANup98) and EGFP-fused VSV M-expressing DNA (pEGFPN3-M) [20] were kindly provided by Dr. Elisa Izaurralde (European Molecular Biology Laboratory). A *Bss*HII-*Xho*I DNA fragment covering the coding region of HA-tagged human Nup98 region was cloned into a site downstream of CMV promoter in pcDNA3.1/Zeo (+) (Invitrogen, Carlsbad, CA, USA) (pcDNup98). Alanine substitutions from Asp-Thr-Tyr at the position of VSV M 52–54 [VSV (M)] were introduced using an oligonucleotide-directed in vitro mutagenesis system (Quickchange site-directed mutagenesis, Stratagene, San Diego, CA, USA). DsRed-fusion recombinant protein with NLS, U1A and rpL23a was produced in *Escherichia coli*. A double-strand synthetic nucleotide of SV40 NLS (5′-CCA TGC ATA TGC CAA AAA AGA AGA GAA AGG TTG-3′) and PCR-amplified DNA fragment of U1A (1–486), or rpL23a (1–486) from mRNA of HeLa cells was cloned into the *Sma*I or *Sal*I-*Bam*HI sites of pDsRed1-N1 (Clontech, Palo Alto, CA, USA), and then a *Sal*I-*Not*I fragment was inserted in the *Sal*I-*Not*I site of pGEX-4T-2 (Amersham Pharmacia Biotech, Piscataway, NJ, USA). *E. coli* ER2566 (New England Biolabs Inc., Beverly, MA, USA) was used, and recombinant proteins were purified on glutathione sepharose 4 Fast Flow (Amersham) by standard protocols, as previously described [21].

2.6. Reverse-transcription PCR

Total RNA was extracted from transiently transfected cells by using a RNeasy RNA-preparation Kit (Qiagen, KJ

Venlo, The Netherlands). Reverse transcription and PCR were carried out using a SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen). We used the following primers to detect the specific transcripts: for Nup107, 5′-AAACGCGGTAGCTAAACTGCA-3′, 5′-ACCACCAGCTGACTTTGTCGA-3′; for Nup214, 5′-CTTGCCACGAAAACCGTGA-3′, 5′-CAACCCGCAGTCCTGAAAA-3′; for p62, 5′-CAGACACCGACGGATTTGCTT-3′, 5′-TGGATGTTGTTGTGGAGGTGC-3′; for Nup98, 5′-TCTCATCCCAAAACAATGCCTT-3′, 5′-AAACAAAGATGCCTGTCCAGCA-3′; for Nup153, 5′-TGACAATGAAGAGCCAAAGTGT-3′, 5′-TAGGAGTTGTTCCAGAGCCAAA-3′. TaqMan GAPDH Control Reagents (PE-Applied Biosystems) were used as primer sets for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Fifty nanograms of template RNA and 10 pmol of specific primers were used. The efficiency of PCR amplification was roughly in the linear range, as determined by preliminary test with increasing number of cycles. Finally, the PCR products were analyzed by agarose gel electrophoresis using standard techniques.

2.7. Virus vector infection

For HIV-1 vector preparation, a replication-incompetent EGFP-expressing lentivirus (pCS-CDF-CG-PRE) or siRNA-expressing lentivirus was co-transfected into 293T cells along with VSV G-expressing plasmid (pVSV G), HIV-Gag-Pol-expressing plasmid (pRRE) and Rev-expressing plasmid (pRSV-Rev) as described before [6,19]. Three days after transfection, the culture supernatants were cleared by filtration and concentrated through centrifugation at 6000 × *g* for 16 h at 4 °C. The transducing unit (TU) was determined by measurement of EGFP-, or H-2K^k-expressing cells using flow cytometry. Phycoerythrin-labeled anti-mouse H-2K^k monoclonal antibody (mAb) (Cedarlane, Ontario, Canada) was used. Cells were analyzed on FACS SCAN, using Cell Quest software (BD PharMingen, San Diego, CA, USA). Treatment with DNaseI (20 μg/ml) was performed to remove plasmid DNA in the virus stocks. Heat-inactivated (65 °C, 30 min) virus liquid was used as negative control for HIV DNA quantification in infected cells. APH-treated MT-2 cells or 293T cells (2×10^5 cells) were infected with HIV-1 vector (4×10^5 TU). Two hundred thousand 293T cells were transfected with VSV M- or Nup98-expressing DNA and then 24 h later, infected with HIV-1 vector (4×10^5 TU). Two hundred thousand 293T cells were transfected with siRNA-expressing DNA and then 72 h later, infected with HIV-1 vector (4×10^5 TU). The amount of viral DNA was measured by the quantitative PCR assay 24 h after infection, as described above. HeLa cells (1×10^5 cells), grown on cover-slips, were infected with siRNA-expressing HIV-1 vector at multiplicity of infection (m.o.i.) of 1. The cells were analyzed 96 h later by immunofluorescence, immunoblotting or nuclear import assay.

2.8. Immunofluorescence analysis

HeLa cells, grown on cover-slips, were washed twice with phosphate-buffered solution (PBS) and fixed in 4% (vol/vol) paraformaldehyde/PBS for 15 min at room temperature. The cells were permeabilized with 0.2% Triton X-100/PBS for 5 min. After blocking with 5% bovine serum albumin (BSA)/0.1% Triton X/PBS for 1 h, the cells were incubated with an NPC-specific mouse mAb, mAb414 (BabCO, Berkeley, CA, USA) or anti-Nup98 polyclonal antibody (C-16) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4 °C overnight. Cells were washed three times with 0.05% Triton X/PBS and then incubated with Alexa 594-conjugated goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR, USA) or fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG antibodies (Chemicon, Temecula, CA, USA) for 1 h. Cells were washed three times with 0.05% Triton X/PBS, mounted in Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA, USA) and analyzed with a Leica QFluoro system. The cells were also stained with Hoechst 33342 (Molecular Probes).

2.9. Nuclear import assay

HeLa cells grown on cover-slips were washed in PBS and permeabilized for 5 min on ice in 50 μg/ml digitonin (Sigma)/transport buffer (20 mM Hepes– NaOH, pH 7.3, 110 mM CH_3COOK, 2 mM $(CH_3COO)_2Mg$, 5 mM CH_3COONa, and 2 mM dithiothreitol). After washing three times with transport buffer, cells were incubated at 30 °C for 30 min in the presence of energy-regenerating system (1 mM ATP, 1 mM GTP, 10 mM creatine phosphate, and 20 U/ml creatine phosphokinase), 3 μM DsRed-labeled recombinant protein, and cytoplasmic extract from 2×10^5 HeLa cells. Samples were washed three times in transport buffer, fixed on ice for 30 min with 1% formalin/transport buffer and analyzed with a Leica QFluoro system.

2.10. Immunoblotting

293T cells were co-transfected with an HA-tagged human Nup98-expressing plasmid DNA (pcDNup98) and a siRNA-expressing plasmid (siN98 or siLuc). Three days after transfection, the cells were washed twice and lysed in RIPA buffer (0.5% NP-40 in 20 mM Tris–HCl [pH 8.2], 0.15 M NaCl, 5 mM iodoacetamide, and 1 mM phenylmethylsulfonyl fluoride). After loading on SDS/PAGE, polypeptides were transferred to Immobilion Transfer Membranes (Millipore, Billerica, MA, USA), the level of Nup98 was determined using a goat anti-Nup98 polyclonal antibody (C-16), biotin-conjugated rabbit anti-goat IgG (Chemicon) and then incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Zymed, San Francisco, CA, USA). The filter generated from HeLa cells infected with siRNA-expressing

HIV-1 vector as described above was also incubated with mAb414 (mainly reactive against p62), biotin-conjugated horse anti-mouse IgG (VECTOR) and HRP-conjugated streptavidin. The specific bands were detected using Western Lighting Chemiluminescence Reagent (Perkin–Elmer Life Science, Boston, MA, USA). For detection of Nup98-VSV M complex, 293T cells were co-transfected with Nup98-expressing plasmids (pcDNup98) and EGFP-fused VSV M-expressing plasmid DNA (pEGFPN3-M) or the mutant [VSV M(D)]. Two days after transfection, the cells were lysed in triple detergent lysis buffer (1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate in 50 mM Tris–HCl [pH 8.0], 0.15 M NaCl, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), and a mouse anti-HA mAb (F-7) (Santa Cruz) was added. After incubation for 12 h at 4 °C with protein G-sepharose (Amersham), the precipitate was washed three times with triple detergent lysis buffer, and the bound proteins were eluted by 1× sample buffer (1.71% SDS in 175 mM Tris–HCl [pH 6.8], 5% glycerol, 1% 2-mercaptoethanol) at 37 °C for 30 min. The samples were loaded on SDS/PAGE and transferred to Immobilon Transfer Membranes. For detection of the Nup98, a goat anti-Nup98 polyclonal antibody (C-16) (Santa Cruz) and biotin-conjugated rabbit anti-goat IgG (Chemicon) were used. For detection of VSV M, a rabbit anti-GFP polyclonal antibody (Santa Cruz) and biotin-conjugated donkey anti-rabbit IgG (Chemicon) were used.

2.11. Statistical analysis

All data were expressed as mean ± standard deviations (S.D.). Differences between groups were examined for statistical significance using the Welch's *t*-test. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

3. Results

3.1. Efficient nuclear import of HIV-1 cDNA in infected cells

It has been shown that HIV and HIV-based lentivirus vectors efficiently infect non-dividing cells [2,6]. To determine the integration efficiency in dividing and non-dividing cells, we prepared cell-cycle-arrested T cell culture using MT-2 cells treated with APH, an inhibitor of DNA polymerase α. Under this condition, cell-cycle was confirmed to be stopped at G1 phase from flow cytometric analysis (Fig. 1A). The same numbers of treated (arrested) or untreated (proliferating) cells were infected with the same amounts of HIV-1 vector and the arrested culture was further maintained in the presence of APH. Since in this experiment we used a single-round infection system, we could estimate the precise efficiency of reverse transcription, nuclear translocation as well as integration. Total DNA was extracted 24 h after infection and a set of real-time PCR assay was performed [16,17]. Using this assay, we were able to measure the full-length/1LTR circle, 2LTR circle and integrated forms of HIV-1 cDNA, respectively. Since the 2LTR circle and integrated forms are found only in nucleus after HIV infection [3,22], we could estimate the efficiency of nuclear entry as well as integration of HIV-1 cDNA. Fig. 1B shows that the levels of integrated, 2LTR and full-length/1LTR circle form in proliferating cultures were higher than those in arrested cultures, because the numbers of the cells were two to three times greater in proliferating culture. However, significant amounts of integrated ($4.2 \times 10^5 \pm 5.4 \times 10^4$ copies per culture) and 2LTR ($1.5 \times 10^5 \pm 1.5 \times 10^3$ copies per culture) form DNA were found in the arrested culture (Fig. 1B). Similar results were also obtained in APH-treated 293T cells (data not shown). These data correspond well with the previously reported findings of the high susceptibility of APH-treated cells to HIV-1 [3,22]. Importantly, the ratios of integrated form and 2LTR form to full-length/1LTR form were similar in the proliferating (integrated; 0.108 ± 0.024, 2LTR; 0.022 ± 0.002, full-length/1LTR; 1.0) and the arrested cultures (integrated; 0.09 ± 0.011, 2LTR; 0.031 ± 0.001, full-length/1LTR; 1.0), respectively, strongly suggesting that HIV-1 cDNA efficiently traverse NPC, depending on the active nuclear import machinery in not only non-dividing cells but also proliferating cells.

3.2. Inhibition of HIV-1 cDNA import with a Nup98-specific inhibitor

Next, to examine the specificity of our real-time PCR assay and the associated molecules in nuclear entry of HIV-1 cDNA, we prepared HIV reverse transcription-inhibited (AZT-treated), transcription-blocked (ActD-treated), or CRM1-dependent nuclear export-inhibited (LMB-treated) 293T cell cultures. As expected, AZT significantly inhibited the appearance of all forms of DNA (Fig. 2A a–c). Although dose-dependent inhibition of integration was found in ActD- or LMB-treated cultures, significant accumulation of the 2LTR form was also found (Fig. 2A f and i), suggesting that newly synthesized proteins as well as CRM1-dependent exported proteins may be required for the efficient integration but not nuclear entry of HIV-1 cDNA. To examine whether specific Nups are required for HIV infection, we used VSV M protein, a specific inhibitor protein against Nup98. It has been shown that Nup98 is involved in the nuclear import of some proteins as well as the export of RNA, and its function is specifically impaired in the presence of VSV M protein [20]. The VSV M binds a region within residues 66–515 of Nup98 that encompasses most of the FG repeats, the hRAE1/Gle2 binding site or GLEBS-like motif [23], and most of the predicted glycosylation sites of the Nups. Through these sites, Nup98 was able to interact with three

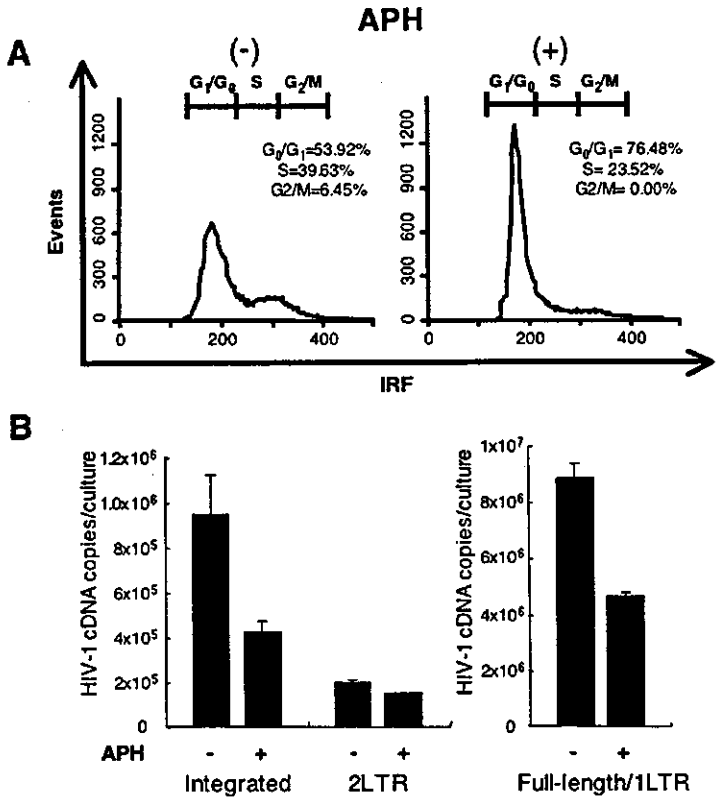

Fig. 1. Efficient nuclear entry of HIV-1 cDNA in arrested cells. (A) Cell-cycle analysis of APH-treated and -untreated cells. MT-2 cells were incubated for 24 h without (–) or with (+) APH, and then analyzed for DNA content by flow cytometry of proridium iodide-stained nuclei. Representative flow cytometry data from one of three independent experiments is shown. (B) Quantification of HIV-1 cDNA after HIV-1 vector infection in APH-treated and -untreated cells. Number of viral DNA copies per culture (baseline cell number is 2×10^5 cells) is indicated. APH-treated or -untreated MT-2 cells were infected with HIV-1 vector, and cultured without (–) or with (+) APH for another 24 h, respectively. Then, DNA was extracted and subjected to PCR assay. Results are mean ± S.D. of three independent experiments.

putative Nup98 partners: RAE1 [23], CRM1 [24], and TAP [25]. VSV M-mediated inhibition was not observed in a site-directed mutant (residue 52–54), termed VSV M(D) [20]. We used the VSV M as a specific inhibitor of the Nup98 function. An obvious impairment of integrated and 2LTR but not full-length DNA was found in only the wild-type but not the mutant VSV M(D)-transfected culture (Fig. 2B). This impairment was restored with ectopic overexpression of Nup98 (pcDNup98) (Fig. 2B, lane 5). Western blotting indicated that the overexpressed Nup98 was co-precipitated with VSV M but not VSV M(D) protein (Fig. 2C), suggesting that the overexpressed Nup98 absorbed VSV M protein, and the Nup98 function was recovered. Thus, Nup98 may have a role in nuclear import of HIV cDNA.

3.3. Depletion of Nup98 by siRNA

Next, to examine directly the involvement of Nup98 in HIV-1 cDNA nuclear import, Nup98 was depleted by the siRNA technique. After transfection with Nup98-specific siRNA-expressing plasmid, mRNA expression of Nup98 as well as Nup96, generated from the same precursor transcripts of Nup98 [26], but not other Nups such as p62, Nup107, Nup153, and Nup214, were specifically inhibited (Fig. 3A). It was also confirmed that the level of ectopic Nup98 protein expression was inhibited with the siRNA-expressing plasmid as it was lower than that in its endogenous expression (Fig. 3B). Immunofluorescence analysis using an anti-Nup98 antibody also confirmed the significant inhibition of Nup98 expression on nuclear membrane in the Nup98 siRNA-targeted HeLa cells using a siRNA-expressing lentivirus vector (Fig. 3C, upper panel). We further examined the distribution of NPC components using mAb414, an antibody known to interact with many FG-containing Nups, mainly p62 and to a less degree, Nup153, Nup214, and Nup358 but not Nup98. The Nup98 siRNA-transduced cells exhibited weak mAb414-labeling intensity at the nuclear rim and shift of labeling to the cytoplasm, probably cytoplasmic annulate lamellae (Fig. 3C, lower panel). However, the total amount of p62 (main component of NPC) was similar in both Nup98-siRNA-targeted or control cultures (Fig. 3D). A previous study using Nup98 knockout mice indicated that Nup98 is essential for rapid cell proliferation but dispensable for basal cell growth and some specific destruction of NPC component [27]. The Nup98-knockout cell was reported to have a thin nuclear envelope as well as many cytoplasmic annulate lamellae. Our Nup98-siRNA-targeted cells had similar structures. It was also reported that the mutant pores of the knockout cells were clearly impaired in in vitro transport

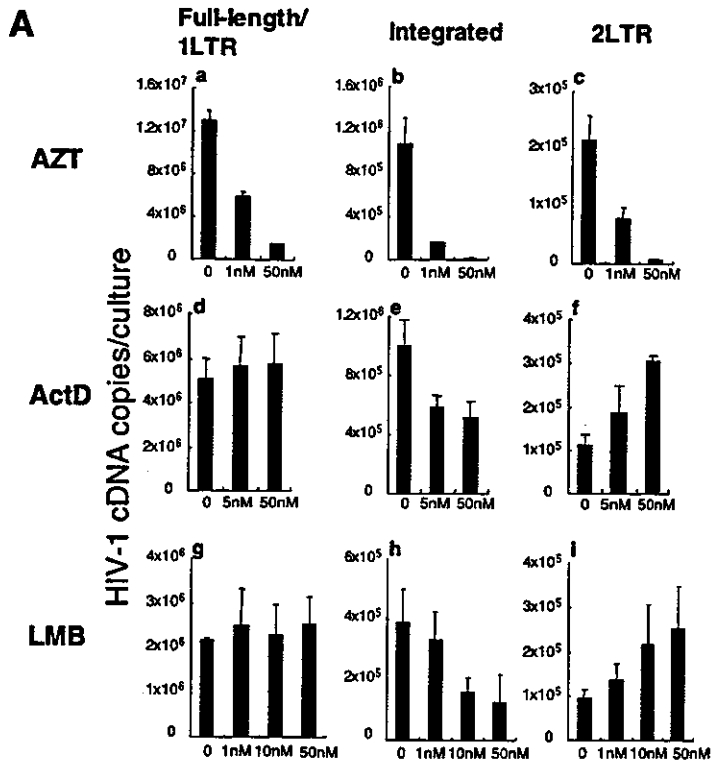

Fig. 2. Measurement of nuclear entry and integration of HIV-1 cDNA in cells treated with inhibitors. (A) Inhibition of HIV-1 cDNA appearance with chemical treatment. 293T cells were infected with HIV-1 vector at a m.o.i. of 0.5–1 and cultured in the presence of AZT, ActD, or LMB at indicated doses. HIV-1 cDNA copy numbers in AZT-treated (a, b, c), ActD-treated (d, e, f) and LMB-treated (g, h, i) cultures are indicated. Left panels (a, d, g) indicate full-length/1LTR circle, middle panels (b, e, h) indicate integrated forms, and right panels (c, f, i) indicate 2LTR circle, respectively. (B) Inhibition of nuclear entry of HIV-1 cDNA with VSV M transduction. Full-length/1LTR circle, 2LTR circle and integrated forms of HIV-1 cDNA in VSV M-transduced cultures are indicated. 293T cells were transfected with VSV M- or VSV M(D)-expressing DNA (lane 3, 4). pcDNA3.1/Zeo(+) was used as control (lane 2). The VSV M-transduced cells were also transfected together with Nup98-expressing DNA (lane 5). Then, these cells were infected with HIV-1 vector 24 h later. Total DNA was extracted 24 h after infection. Results are mean ± S.D. of three independent experiments (A and B). $*P < 0.05$ was judged as a significant difference using Welch's t-test. (C) Binding of Nup98 to VSV M protein. Western blotting using anti-GFP antibody (whole lysate) indicates VSV M or VSV M(D) expression. Binding of ectopically expressed Nup98 to VSV M protein was shown by immunoprecipitation with anti-HA antibody (anti-HA IP) and Western blotting using anti-Nup98 or anti-GFP antibodies. Results of one representative experiment from three independent experiments are shown.

assays with nuclear localization signal (NLS) of SV40 or M9 import signal (mediated by importin α/β and importin β2, respectively), while the ability of the mutant pore to import ribosomal protein L23a (mediated by either importin β, importin β2, importin β3 or importin 7) [28] and splice some protein U1A (independent of cytosolic transport factors) was intact [27]. To examine the import ability of Nup98-siRNA-targeted pores, we performed a set of import assays. These experiments were performed in a transport buffer containing specific soluble transport receptors, an energy-regenerating system, and a DsRed-labeled protein acting as a substrate for import into the nuclei of digitonin-permeabilized cells [29]. The nuclear import with an SV40 NLS import signal was substantially lower in the Nup98-depleted cells than in control cells. In contrast, translocation of rpL23 or U1A was similar in Nup98-depleted and control cells (Fig. 2E, F). These results indicated that protein import pathways were similarly impaired in both Nup98-depleted human cells using siRNA and Nup98-knockout cells, although the level of impairment of NLS in the Nup98-depeleted cells using siRNA seems to be lower than that in knockout cells.

3.4. A role of Nup98 in HIV-1 cDNA import

Finally, Nup98-depleted cells using a siRNA-expressing plasmid DNA were infected with HIV-1 vector, and the levels of integrated, 2LTR, and full-length/1LTR forms of HIV-1 cDNA were measured. Obvious reductions of integrated and 2LTR but not full-length DNA were noted in the Nup98-depleted culture. In contrast, in culture transfected with a control siRNA-expressing DNA targeted for luciferase (si-Luc), the levels of integrated, 2LTR and full-length DNA were still high (Fig. 3G). These findings suggest that Nup98 in the NPC participates in the nuclear entry of HIV-1 cDNA.

4. Discussion

The major finding of the present study is that Nup98 has an important role in the nuclear import of HIV-1 cDNA, based on a series of experiments using an inhibitor (VSV M protein) and the siRNA technique for Nup98. The role of

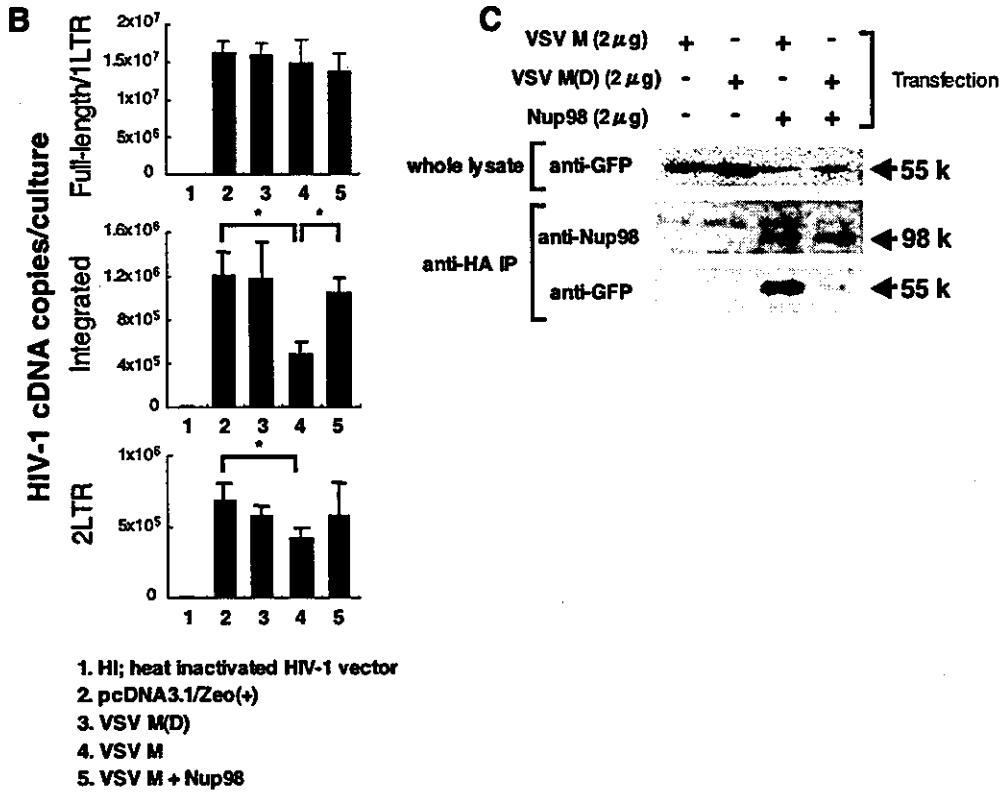

Fig. 2. *(continued)*

NPC in virus replication has been shown previously in other virus families [30]. It was reported that the import process of adenovirus type 2 DNA through NPC involves Nup214 as well as histone H1 [31]. Adenovirus capsid docks on Nup214, probably involving recruitment of importin 7/β heterodimer to the capsid, and effective disassembly at NPC activates the entry of viral DNA into nuclei [31]. Herpes simplex virus capsid docks on NPC, probably mediated by importin β alone, and undergoes a conformational shift that results in extrusion of viral DNA genome into nucleus through NPC [32]. It is possible that specific molecular events may occur at NPC during the nuclear entry of HIV-1 cDNA [15].

We recently transduced a siRNA-expressing DNA for Nup98 using lentivirus vector coexpressed H-2K^k, as described above, and at this time the culture was challenged with replication-competent EGFP-expressing HIV-1. Flow cytometric analysis indicated that numbers of HIV-1-infected cells were obviously inhibited in Nup98-siRNA-expressing T cells but not in control luciferase-specific siRNA-expressing cells. Significant reduction of HIV-1 p24^{gag} (five to sixfold) was also noted in the culture supernatant (data not shown). It was reported that Nup98 and Nup214 are required for Rev-dependent export of HIV-1 RNA [24]. Thus, inhibition of Nup98 expression by functional impairment of Nup98 by siRNA may induce inhibition of HIV-1 gene expression through inhibition of the Rev-dependent export. Some degree of the inhibitory effect of HIV-1 infection by the siRNA may be partly caused by this inhibitory effect of Rev function. But quantitative analysis of HIV-1 cDNA in single-round infection demonstrated that the inhibition of nuclear import of HIV-1 cDNA (Fig. 3G) was apart from gene expression. These findings suggest the involvement of a specific import pathway in the nuclear entry of HIV cDNA through NPC.

Most of the transport receptors identified to date are members of a large family of RanGTP binding proteins, which exhibit a limited sequence similarity to the Ran binding domain of importin β. The interaction of these receptors with Nups is regulated by small GTPase Ran. Ran is a small GTPase that cycles between a GDP-bound form (RanGDP) and a GTP-bound form (RanGTP) and plays an important role in both import and export [8]. The directional active nuclear transport is controlled by the different RanGDP and RanGTP concentration gradients within the cell. In the cytoplasm, a much higher concentration of RanGDP to RanGTP is maintained, and conversion of RanGDP to RanGTP occurs by exchanging the entire nucleotide and is catalyzed by the guanine nucleotide exchanging factor (RCC1) [33]. The exchange of the nucleotide and disassembly of importin β2 complex at a site on Nup98 was reported [13]. The VSV M-mediated inhibition of nuclear traffic is due to the inhibition of RanGDP to RanGTP conversion [13]. Therefore, in the presence of VSV M expression or inhibition of Nup98 expression should induce the disruption of the RanGDP and RanGTP concentration gradients. Our data strongly suggest

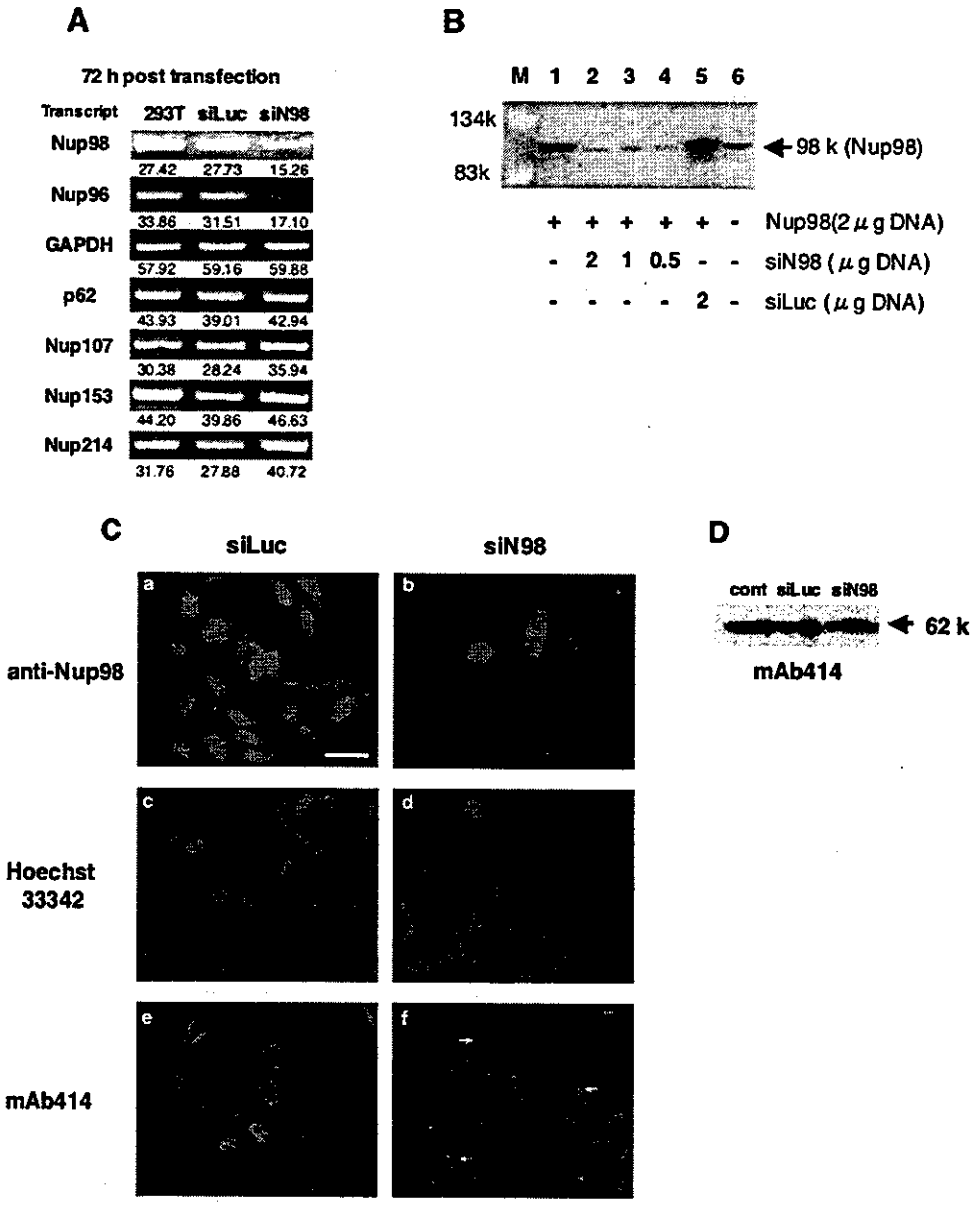

Fig. 3. Depletion of Nup98 by siRNA impairs specific NPC function. (A) Inhibition of Nup98 and Nup96 mRNA expression with Nup98-targeted siRNA. 293T cells were transfected with siRNA-expressing plasmid DNA targeted for Nup98 or luciferase (siN98 or siLuc, respectively) and mRNA levels of Nup98, Nup96, p62, Nup107, Nup153, Nup214 or GAPDH were determined by RT-PCR. The numbers under the band indicate intensity of each band digitalized by using NIH image. (B) Inhibition of Nup98 expression with siRNA-expressing plasmid DNA. Western blotting analysis using an anti-Nup98 antibody in siLuc or siN98-transfected 293T cells is indicated. Nup98-expressing plasmid (Nup98 as indicated) was transfected without (lane 1) or with (lane 2-5) siRNA-expressing plasmid as indicated. The expression level of endogenous Nup98 is indicated in lane 6. Size marker (M) is indicated. (C) Expression of Nup98 and localization of FG-repeat nucleoporins in siRNA-transduced cultures. Immunofluorescence analysis of HeLa cells infected with siLuc (a, c, e)- or siN98 (b, d, f)-expressing lentivirus vector using an anti-Nup98 (upper panels) or mAb414 antibody (lower panels) are shown. Hoechst 33342 staining (middle panels) indicates nucleus. Blue arrow in bottom panel indicates weak signal on nuclear rim, and white arrow indicates a shift labeling to the cytoplasm, probably annulate lamellae form. Calibration bar = 30 μm. (D) Western blotting analysis of HeLa cells infected with siRNA-expressing lentivirus vector using mAb414. The level of p62 protein expression is indicated. (E) Nuclear import assay of Nup98-depeleted HeLa cells. Cells were infected with the siLuc (upper panels)- or siN98 (lower panels)-expressing lentivirus vector, and 3 days later, nuclear import for these depleted cells after permealization with digitonin was examined using DsRed-labeled SV40 NLS (a, d), U1A (b, e), and rpL23a (c, f), respectively. Import of SV40 NLS is dependent on importin α/β and that of rpL23a is dependent on importin β3. Import of U1A is independent of active transfer machinery. The reaction was performed at 30 °C and stopped after 30 min by fixation. Calibration bar = 30 μm. Results of one representative assay from three independent experiments are shown (A, B, C, D, E). (F) Quantification of nuclear import. Cells with nuclear import were counted in five randomly selected visual fields. Data are expressed as percentage of imported cells (mean ± S.D. of five fields). *$P < 0.05$, compared with siLuc by Welch's t-test. (G) Measurement of newly synthesized HIV-1 cDNA in siLuc- or siN98-transfected cells. 293T cells were transfected with siN98- or siLuc-expressing plasmid DNA, and 72 h later, EGFP-expressing HIV-1 vector was used for infection. DNA was extracted 24 h after infection and DNA was subjected to real-time PCR assay. The number of HIV-1 cDNA copies per culture is indicated. A heat inactivated HIV-1 vector (HI) was used as negative control. Results are mean ± S.D. of six independent experiments. *$P < 0.05$, compared with siLuc by Welch's t-test.

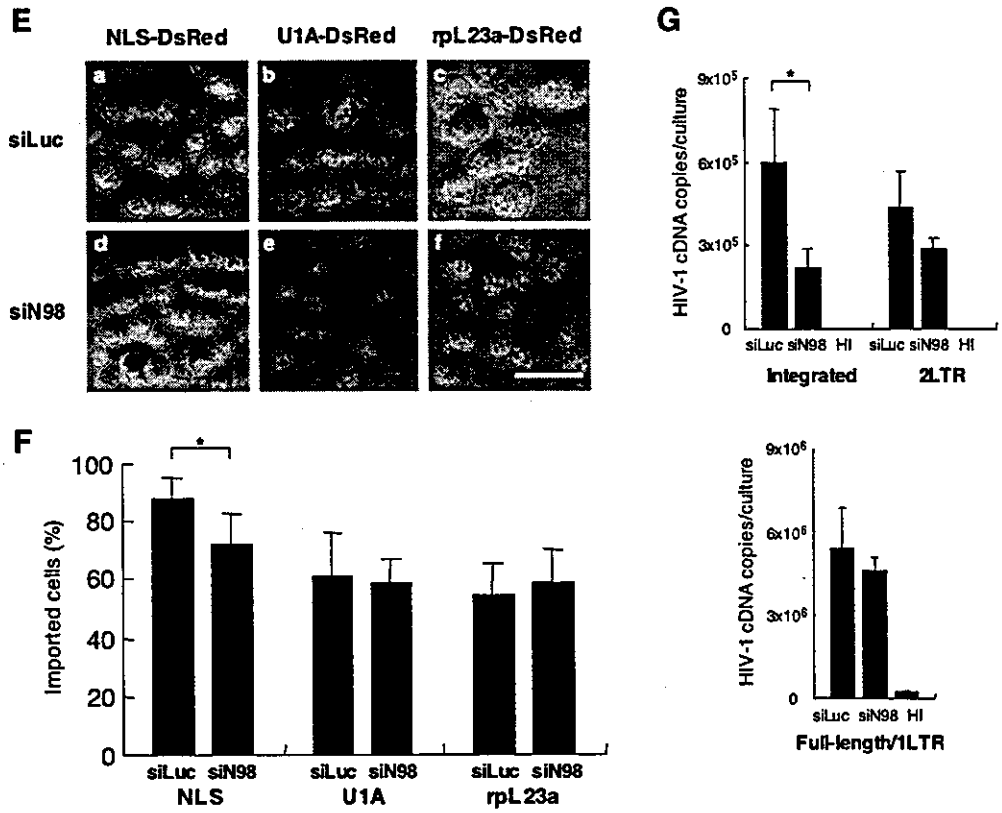

Fig. 3. *(continued)*

that these RanGTP/GDP gradients and localization of Nups are important for the directional active transport of HIV-1 cDNA. In fact, it has been recently reported that importin 7 is a major transport receptor for nuclear import of HIV-1 PIC [15]. Fassati et al. showed that PIC isolated from HIV-1-infected macrophages efficiently entered the nucleus mediated by importin 7 in a Ran- and energy-dependent manner.

Importin 7 is also known as a transport receptor for ribosomal proteins and histone H1 [28,34]. In our experiments, Nup98 depletion hampered the nuclear entry ability mediated by importin α/β, which was examined by NLS. In contrast, the nuclear entry abilities predominantly mediated by importin β3, which was examined by rpL23a, and these transporter-independent pathways, which were examined by U1A, were not affected in the Nup98-depleted NPC. Import of rpL23a was mediated alternatively by multiple transport receptors such as importin β, importin β2 (transportin), importin β3 and importin 7 [28]. It was reported that the mutant pore of Nup98-knockout cells has reduced affinity for many specific transport receptors, especially importin 7 [27]. Thus, it is possible that the functional association of importin 7 and Nup98 may disrupt and Nup98 depletion may induce impairment of the nuclear import pathway predominantly mediated by importin 7. In the present study, we did not succeed in establishing in vitro nuclear import assay of HIV-1 PIC using Nup98-depleted NPC. It will be very interesting to examine whether Nup98 is directly involved in the nuclear import of PIC. Further biochemical analyses, such as experiments showing large complex formation with PIC, importin 7, and Nup98 in infected cells are required.

In conclusion, we have demonstrated in the present study the role of Nup98 in the nuclear entry of HIV-1 cDNA in cultured cells. Our results suggest that a similar mechanism may be operative in HIV-1-infected individuals. Therefore, inhibition of Nup98 function could be a potentially important target for therapeutic intervention in patients with acquired immunodeficiency syndrome.

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A Lentiviral cDNA Library Employing Lambda Recombination Used To Clone an Inhibitor of Human Immunodeficiency Virus Type 1-Induced Cell Death

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Expression cloning technology of cDNAs is a suitable tool for identifying novel functional properties of genes. Here, we generated a lentiviral cDNA library-expressing system for human T cells based on a site-specific recombination system of phage lambda for transferring cDNA libraries with a minimum loss of its complexity. The library-transduced CD4$^+$ T cells were challenged with wild-type human immunodeficiency virus type 1 (HIV-1), and the cells that acquired resistance to HIV-1-induced cytopathic effect (CPE) were selected. From these cells, CD14 was isolated and proved to inhibit the entry of HIV-1 and the HIV-1-induced CPE. This cloning system allows rapid identification of genes encoding novel properties in human T cells and probably other mammalian cells.

A number of screening systems from genetic libraries have been developed to identify novel functional properties of the genes. A successful screening with mammalian cells is dependent on the efficiency of the transduction system into the appropriate target cells. A plasmid-based expression system has been generally used (1, 2, 17). However, this system has a limit due to the inefficient transfection into particular cells, such as nonadherent cells. In addition, the introduced genes are expressed only transiently. Therefore, it is desirable to develop a new technology that can efficiently achieve long-lasting expression of genetic information in the nonadherent cells, especially human lymphocytes. Retrovirus vectors appear to overcome these limits (16). Retrovirus infects a wide range of mammalian cell types, including lymphocytes, with a high efficiency. The library-inserted retrovirus vector can integrate into the host's chromosome and is expressed permanently. These properties have been utilized for a gene delivery system for lymphocytes (16). However, the prototype murine leukemia virus-based retrovirus vector infects only dividing cells (12) and, less efficiently, human T cells. Thus, the target cells for screening are limited. Recently, a human immunodeficiency virus (HIV)-based lentivirus vector was developed (12), and such vectors are beginning to be used in many applications (4, 7, 10, 11).

In this report, we describe the development of a lentiviral cDNA library expression system applicable for human T cells. The results showed significant utility of the system to clone genes through a high-throughput screening procedure. This system allowed us to identify genes that render cells resistant to HIV-induced cell death. Our lentivirus system is promising, as it can be applied to many library screening systems, and should accelerate the discovery of novel properties of the genes in many other cells including neurons and hematopoietic stem cells.

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MATERIALS AND METHODS

Cells. Human 293T were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum, and MT-4 cells were maintained in RPMI 1640 containing 10% fetal calf serum.

Gateway-compatible lentiviral cDNA library system and HIV-1 challenge. A Gateway-compatible lentivirus vector DNA (pYK005C) was constructed through the insertion of a Gateway cloning system reading frame cassette (Invitrogen, Carlsbad, Calif.) into the EcoRI site of the multiple cloning sites (MCS) in the HIV-1-based vector DNA, pCSII-elongation factor 1α promoter (EF)-MCS-internal ribosome entry site (IRES)-humanized *Renilla* green fluorescent protein (hrGFP) (9). For the generation of the entry cDNA library, 10 ng (~1.5×10^9 copies) of the original cDNA library generated from human peripheral blood leukocytes (Invitrogen) was amplified by PCR with the following primers: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′ (underlined nucleotides are the *att*B [B1 and B2] sequences in the forward and reverse primers, respectively). The cycling conditions were 94°C for 2 min, 94°C for 15 s, 55°C for 30 s, and 68°C for 5 min for 15 cycles and 68°C for 10 min. PCR products and pDONR201 DNA (Invitrogen) were incubated with BP Clonase enzyme mix (Invitrogen) for 16 h at 25°C by using the procedure recommended by the manufacturer, and the resulting recombinant molecules were transformed in DH5α. The transformants were selected with kanamycin (50 μg/ml), and the resultant entry cDNA library was prepared from pools of transformants. For the generation of the vector cDNA library, 300 ng of the entry cDNA library and 360 ng of pYK005C vector DNA, which is linearized by digestion with EcoRI, were incubated with LR Clonase enzyme mix (Invitrogen) for 19 h at 25°C. All resulting recombinant molecules were transformed in DH5α and selected on plates containing ampicillin (50 μg/ml). The resultant vector cDNA library was prepared from pools of transformants. For preparation of cDNA-expressing lentivirus vector, a vesicular stomatitis virus (VSV)-pseudotyped lentivirus vector was generated via calcium phosphate-mediated transfection of 293T cells as described before (9). Briefly, 1.2×10^7 cells were divided onto six TC dishes (100 × 20; Nunc, Roskilde, Denmark) 24 h before transfection. Seventeen micrograms of Vector cDNA library DNA, 12 μg of HIV Gag-Pol-expressing vector (pMDLg/pRRE), 5 μg of VSV-G protein-expressing vector (pMD-G), and 5 μg of HIV Rev-expressing vector (pRSV-Rev) per dish were cotransfected, then 48 h later, the culture supernatants were collected, and virus particles were concentrated 30-fold by centrifugation at 6,000 × *g* for 16 h. The concentrated viruses were titrated with MT-4 cells. For transduction of the cDNA library into T cells and HIV type 1 (HIV-1) challenge, 1.2×10^7 MT-4 cells were infected with 8×10^6 infectious doses of the

viral cDNA library. Three days later, the cells were challenged with HIV-1_{NL4-3} at a multiplicity of infection (MOI) of 0.05. For recovery of the cDNA sublibrary from surviving cells, MT-4 cells that survived HIV-1 challenge were collected and genomic DNA was extracted. The cDNAs from the surviving cells were amplified by PCR with primers that were used to amplify the original cDNA library as described above. This cDNA sublibrary was transferred to the pDONR201 vector by a BP reaction, and the resultant entry cDNA sublibrary was transferred to pYK005C lentivirus vector DNA by an LR reaction as described above. The viral cDNA sublibrary was prepared via transfection of 293T cells and used for the second round of screening.

Flow cytometric analysis. Two-color flow cytometric analysis was performed. Briefly, cells were stained with the optimal concentration of antibody for 30 min at 4°C and then washed. Phycoerythrin-conjugated anti-human CD4 and CD14 (eBioscience, San Diego, Calif.) and anti-mouse *H-2K*^{k} (Cedarlane, Ontario, Canada) were used. HIV-1 expression was examined with an anti-HIV-1 human serum followed by staining with biotin-conjugated anti-human IgG (Vector Laboratories, Burlingame, Calif.) and streptavidin-conjugated peridinin chlorophyll protein (BD Biosciences, San Jose, Calif.). The data were collected by FACScan (BD PharMingen, San Diego, Calif.) and analyzed with WinMDI software.

Sequence analysis. cDNA cloned into the pDONR201 vector was analyzed with the 5'-TCGCGTTAACGCTAGCATGGATCTC-3' primer. The data were collected with the ABI 377 autosequencer. The sequence data were compared with the DNA database at the National Center for Biotechnology Information by using BLAST search.

Determination of individual cDNA length. The original cDNA library, the entry cDNA library, and the vector cDNA library were applied to *Escherichia coli* competent cells, and the cells were spread onto Luria-Bertani plates to develop bacterial colonies. cDNA fragments were amplified by PCR from these bacterial colonies containing each cDNA fragment. The PCR products were subjected to agarose gel electrophoresis and visualized with ethidium bromide. The migration distance of each cDNA fragment was compared with a DNA size marker. MT-4 cells transduced with the viral cDNA library were cloned by the limiting dilution method. cDNA fragments were amplified by PCR from the cloned cells. The length of each cDNA was determined as described above.

CD14 cDNA transduction and HIV-1 infection. A CD14 cDNA-expressing construct was made through the insertion of the Gateway cloning system reading frame cassette (Invitrogen) into the EcoRI site of the pIRES-hrGFP vector (Stratagene, San Diego, Calif.), and then a CD14 cDNA fragment was isolated from the library by an LR reaction. $CD4^+CCR5^+$ HeLa cells (6) were transfected by Lipofectamine 2000 (Invitrogen) with the CD14-expressing construct or empty vector (pIRES-hrGFP) as a control, and then 48 h later, the cells were infected with HIV-1_{NL4-3} at an MOI of 2. Cells were harvested 2, 12, 24, and 48 h after HIV-1 infection, and DNA was extracted as described before (20). For CD14 stable transduction, an *H-2K*^{k}-expressing lentivirus vector, which was constructed by replacing the mutant *Renilla reniformis* hrGFP sequence in the Gateway-compatible lentivirus vector DNA (pYK005C) with the *H-2K*^{k} sequence, was used. MT-4 or $CD4^+$ $CCR5^+$ HeLa cells were infected with either the CD14-expressing or control lentivirus vector at an MOI of 1, and then 2 days later, the cells were challenged with HIV-1_{NL4-3} at an MOI of 0.05. Cell killing activity was measured by trypan blue staining, and virus production in the culture supernatant was monitored by enzyme-linked immunosorbent assay (ZeptoMetrix Corp., Buffalo, N.Y.) for the HIV-1 $p24^{gag}$ antigen.

Real-time PCR assay. For the detection and quantification of individual forms of HIV-1 DNA, strong-stop (early reverse transcript), full-length/1-LTR circle (late reverse transcript), 2-LTR circle, and integrated forms, a real-time PCR assay was used as described previously (20). PCR was performed with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Foster City, Calif.) and *Taq*Man universal PCR master mix (PE Applied Biosystems).

Statistical analysis. The Mann-Whitney U test was used to determine statistical significance, and P values of <0.05 were considered significant.

RESULTS

Transfer of a cDNA library from a cloning expression vector into a donor vector. Since some leukocytes would produce antiviral proteins, we started to isolate anti-HIV genes from a cDNA library generated from human peripheral blood leukocytes. Since a plasmid-based expression vector via transfection cannot be used for efficient and stable transduction into T cells, a lentiviral cDNA library-expressing system was used to introduce genes into human T cells. In this system, we used a recombination-cloning system referred to as Gateway (22). The Gateway system, which has been used to transfer individual genes (22), is based on the recombination system of the phage lambda that mediates integration and excision of the phage DNA into and from the *E. coli* genome, respectively. The integration involves recombination of the *att*P sites (P1 and P2) of the phage DNA within the *att*B sites (B1 and B2) located in the bacterial genome (BP reaction) and generates an integrated phage genome flanked by *att*L (L1 and L2) and *att*R (R1 and R2) sites. The next excision results in these *att*L and *att*R sites back to the *att*P and *att*B sites (LR reaction). First of all, the cDNA library fragments inserted between bacterial genome-derived B1 and B2 sites of the pCMV-SPORT6 cloning expression vector, referred to as the original cDNA library, were amplified by PCR. The PCR product was purified and incubated with a donor vector containing an insertion of the P1 and P2 sites, pDONR201, in the presence of BP clonase enzyme mix, which consists of a mixture of the phage protein integrase (Int) and the bacterial protein integration host factor. The BP clonase recombines the B1 and P1 sites as well as the B2 and P2 sites (BP reaction), and as a result, the cDNA library fragments were placed between derivatives of the L1 and L2 sites. The cDNA library-inserted pDONR201 was referred to as the entry cDNA library. Although a similar number of independent cDNA-carrying clones was obtained after this transfer, the mean size of the cDNA was clearly reduced from 1.75 ± 0.82 kb to 1.34 ± 0.66 kb (± standard deviations [SD]; $n = 60$; $P < 0.05$, Mann-Whitney U test), as shown in Table 1. Generally, the short DNA fragment tends to be more efficiently amplified during PCR. This property may account for the reduction of the cDNA size. However, omission of the PCR resulted in an obvious reduction of the number of independent cDNA-carrying clones by about 1/25. Thus, the PCR amplification before the BP reaction was indispensable.

TABLE 1. Quality of cDNA libraries

cDNA library	No. of primary clones	Mean insert size ± SD (kb)[a]
Original	1×10^7	1.75 ± 0.82
Entry	1.5×10^7	1.34 ± 0.66
Vector	8×10^7	1.26 ± 0.65
Viral	ND[b]	0.71 ± 0.54

[a] Mean insert size was determined by electrophoresis of PCR fragments from 60 bacterial colonies (original, entry, and vector libraries) or from 190 cDNA clones in viral cDNA library-infected cells.
[b] ND, not done.

Transfer of the entry cDNA library into a lentivirus vector. Next, the entry cDNA library was incubated with a lentivirus vector DNA that had derivatives of the R1 and R2 sites in the presence of the LR clonase enzyme mix that consists of a mixture of the phage protein excisionase (Xis), Int, and integration host factor. The LR clonase recombines the L1 and R1 sites as well as the L2 and R2 sites (LR reaction), and DNA fragments between L1 and L2 were placed between the B1 and B2 sites, respectively. Although *cis*-acting sequences derived from the lentivirus vector may interfere with wild-type HIV-1 replication, a part of the vector with a deletion of the U3 region (self-inactivating vectors) was not responsive to the interference (3). Therefore, a self-inactivating vector was used in this study. The cDNA library transferred into the lentivirus

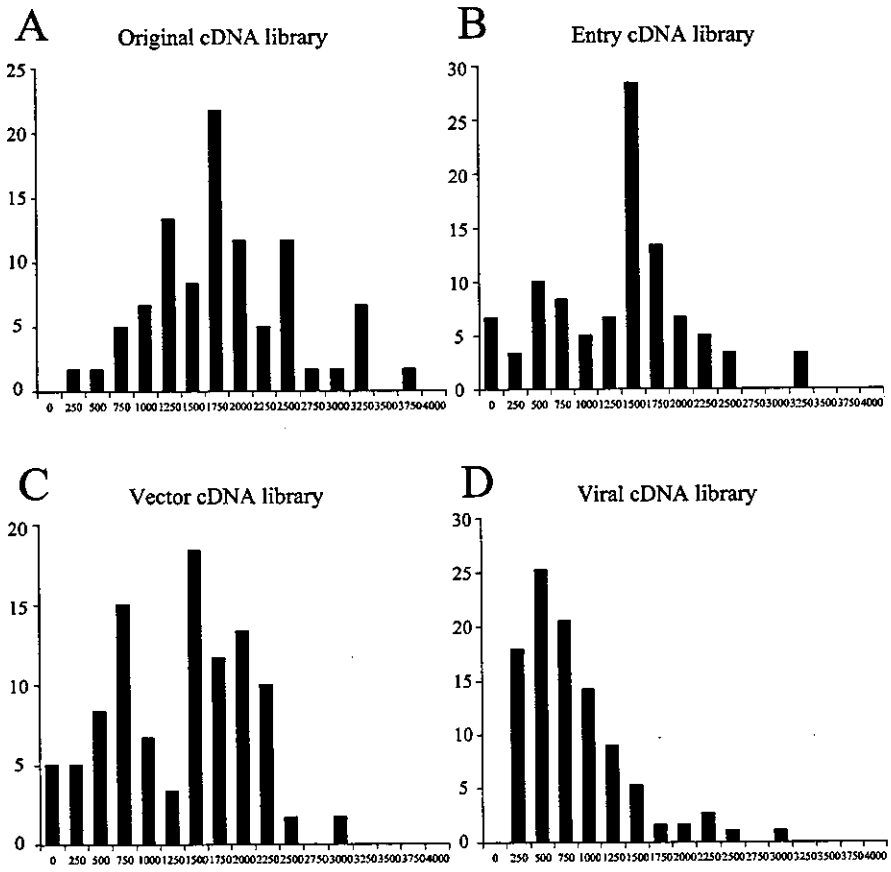


FIG. 1. Histogram analysis of lengths of individual cDNA fragments in each library. The lengths of cDNA fragments were determined as described in Materials and Methods and are plotted in 250-bp increments on the *x* axes. Percentages of individual clones are indicated on the *y* axes.

vector DNA was referred to as the vector cDNA library. Because this lentivirus vector expresses the cDNA library, under the control of elongation factor α promoter, along with GFP expression from a single bicistronic transcript, cDNA library-transduced cells are easily identified by flow cytometry or fluorescent microscopy. After this transfer, no reductions in the number or cDNA size of independent cDNA-carrying clones were observed (Table 1; Fig. 1), suggesting that the library inserted between the L1 and L2 sites would be transferred into another vector without significant loss of library complexity.

Generation of a cDNA library-expressing lentivirus vector. Next we prepared a cDNA library-expressing lentivirus vector, referred to as the viral cDNA library, via cotransfection of 293T cells with vector cDNA library DNA, a VSV-G protein expression DNA, an HIV Gag-Pol expression DNA, and an HIV Rev expression DNA. The infectious titer was approximately 4×10^6/ml, measured by using a human $CD4^+$-T-cell line, MT-4 cells. On the other hand, the infectious titer of the parental lentivirus vector with no cDNA inserted, CSII-EF-MCS-IRES-hrGFP (9), was 10 to 100 times higher than that of the viral cDNA library (data not shown). The average size of cDNA fragments in the transduced cells was 0.7 kb, which was shorter than that of cDNA fragments in the vector cDNA library, suggesting that the smaller cDNAs were enriched during lentivirus preparation and its infection into cells (Table 1; Fig. 1). To overcome this problem, size fractionation to enrich long cDNA fragments should be performed in future experiments. Nevertheless, some transduced cDNAs were more than 2,000 bp (Fig. 1), suggesting that this vector system can transduce more than 2,000-bp cDNA fragments.

Cloning of genes that prevent cells from HIV-1-induced cell death. Figure 2 shows an outline of the selection system used to isolate anti-HIV genes from the library used in this study. Twelve million MT-4 cells were infected with the viral cDNA library at an MOI of approximately 0.68. The total number of cDNA-transduced cells was estimated to be around 8×10^6, which was slightly smaller than the number of independent clones of the original cDNA library. Three days after cDNA transduction, the cells were challenged with $HIV\text{-}1_{NL4\text{-}3}$ at an MOI of 0.05. About 30 days after HIV-1 challenge, when nontransduced culture cells had been completely killed, surviving cells, all of which were continuously growing, and GFP^+ cells were collected and cellular DNA was extracted. The cDNA fragments were recovered by PCR with B1 and B2 primers and transferred into pDONR201 vector DNA through the BP reaction. Then the cDNA sublibrary-expressing lentivirus was generated. After subsequent screening through transduction of the cDNA sublibrary in MT-4 cells and subsequent HIV-1 challenge, more than 25 independent cDNA clones were isolated, which were confirmed in further experiments to

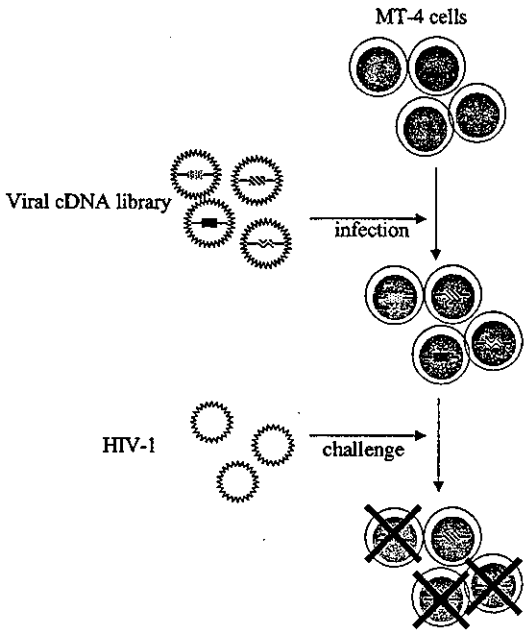


FIG. 2. Scheme for strategy used to select genes that arm cells with resistance to HIV-induced CPE. MT-4 cells were infected with the viral cDNA library and then challenged with HIV-1_{NL4-3}, which is highly cytopathic to MT-4 cells. If the introduced gene has anti-CPE, the cell will survive in the presence of HIV-1.

confer the cytopathic effect (CPE)-free phenotype in the transduced cells after HIV-1 challenge. Sequence analysis revealed that these clones contained full-length CD14 cDNA, and their sequence was identical to that of BC010507 in the GenBank database. Flow cytometric analysis showed that the anti-CD14 antibody reacted only with the cDNA clone-transduced $CD4^+$ cell population (Fig. 3A) identified by GFP expression (Fig. 3B and C).

To verify the effect of CD14 on HIV-1 infection, we independently prepared three cell lines: MT-4 cells transduced with a lentivirus vector that express CD14 along with GFP from a single bicistronic transcript, MT-4 cells transduced with an *H-2K*^{k}-expressing lentivirus vector, and nontransduced MT-4 cells. Flow cytometric analysis confirmed that all GFP-expressing cells simultaneously and persistently expressed CD14 on the cell surface (Fig. 4A). The three cell lines were mixed, and the cultures were challenged with wild-type HIV-1. Before HIV-1 infection, the mixed culture consisted of three cell populations: GFP^+ *H-2K*$^{k-}$, GFP^- *H-2K*$^{k+}$, and GFP^- *H-2K*$^{k-}$ cells (Fig. 4B). Only the GFP^+ *H-2K*$^{k-}$ population survived after HIV-1 infection (Fig. 4C). In contrast, the proportion of the three cell types was consistently maintained in HIV-1-uninfected cultures (Fig. 4D). Trypan blue staining confirmed that all dead cells were GFP^- and all GFP^+ cells were alive (Fig. 4E, F, and G). A subsequent flow cytometric analysis of cells stained by anti-HIV-1 human sera indicated that the CD14-transduced MT-4 cells also expressed HIV-1 antigen (Fig. 4H and I). When the CD14 gene was transduced into human $CD4^+$ $CCR5^+$ HeLa cells, these cells were also susceptible to HIV-1 infection but resistant to HIV-1-induced cell death (data not shown). To reveal the mechanism of how CD14 is blocking the HIV-1-induced cell death, the effect of CD14 for HIV-1 replication was examined. The surface expression of neither CD4 nor CXCR4 was altered in CD14-transduced MT-4 cells (data not shown). On the other hand, the HIV-1 replication in CD14-transduced cells determined by production of $p24^{gag}$ antigen in culture supernatant was significantly lower than that in control vector-transduced cells (Fig. 5A and B). To determine the level at which HIV-1 replication is inhibited by CD14, we used a real-time PCR assay to detect individual forms of viral cDNA at various times after HIV-1 infection. Since the preceding transduction with an HIV-1-based lentiviral vector will hamper the real-time PCR assay to measure the level of newly synthesized HIV-1 cDNA only originated from subsequent wild-type HIV-1 infection, a CD14-expressing plasmid DNA or control vector DNA was

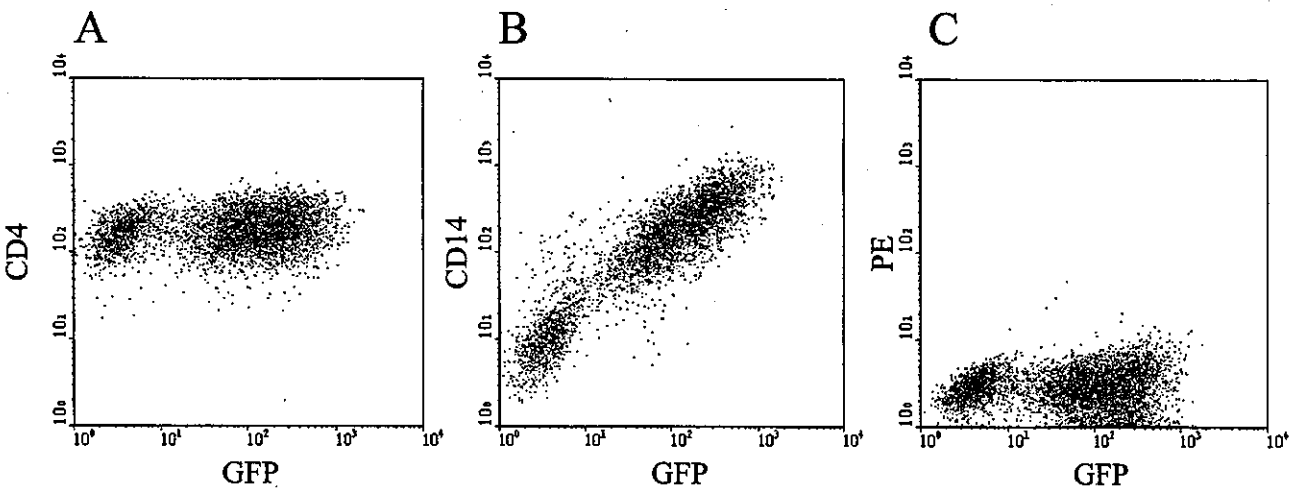
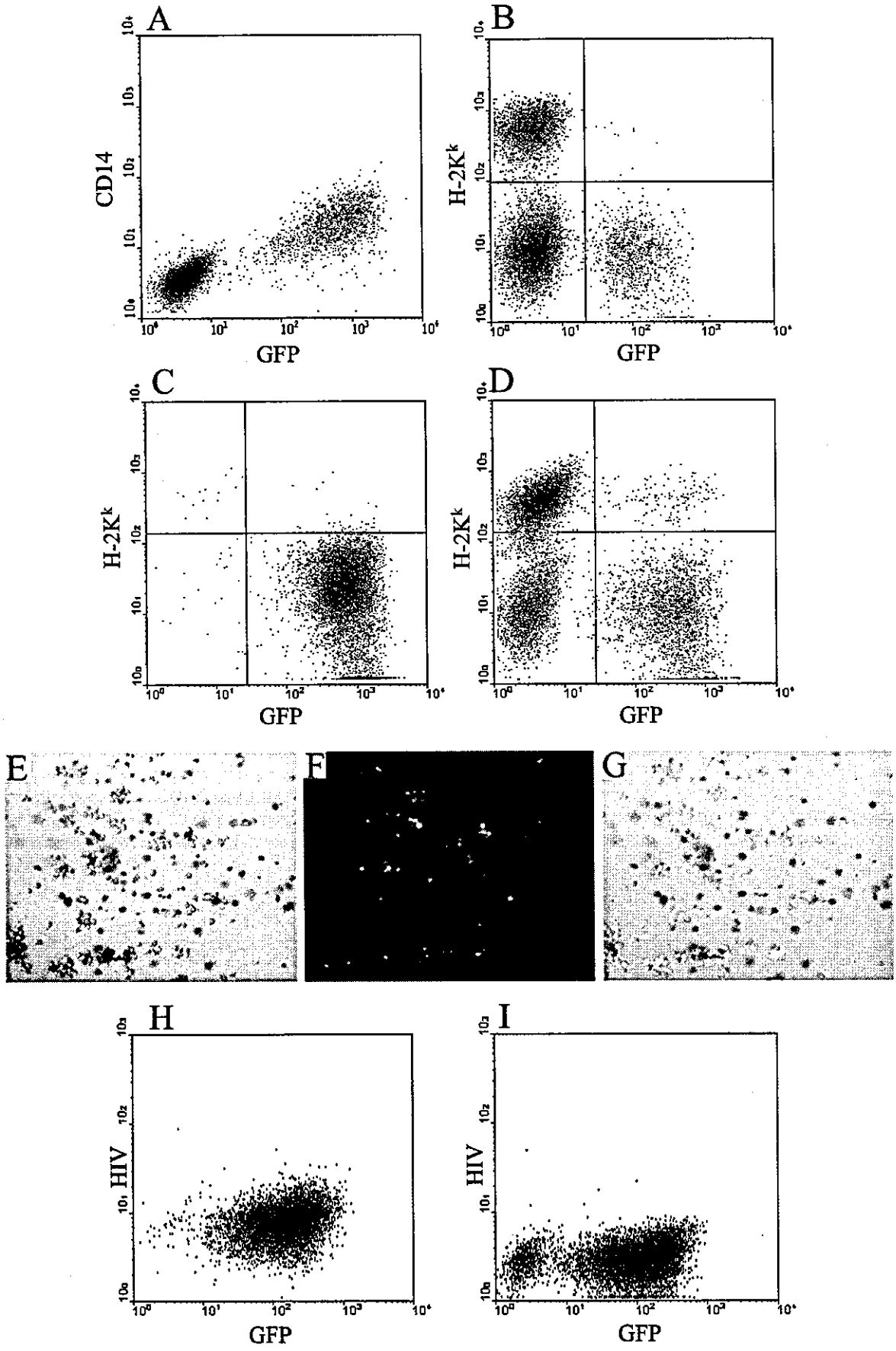


FIG. 3. Characterization of a cDNA clone that confers T-cell resistance to HIV-1-induced CPE. Cells transduced with the CD14-carrying vector isolated from this viral cDNA library were stained with anti-CD4 antibody (A), anti-CD14 antibody (B), or isotype-matched control antibody (C) and analyzed by flow cytometry. The results shown are data from one flow cytometry experiment, which is representative of three independent experiments. PE, phycoerythrin.


A
CD14
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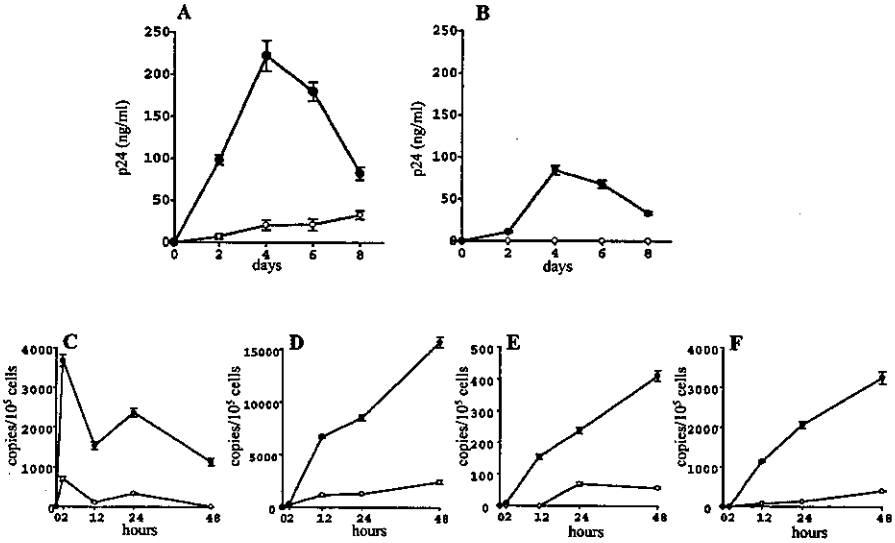

FIG. 5. Inhibition of HIV-1 replication in CD14-transduced cells. HIV-1 replication was evaluated by production of $p24^{gag}$ antigen in the culture supernatant of CD14- or empty vector-transduced MT-4 (A) or $CD4^+$ $CCR5^+$ HeLa cells (B) with a lentivirus vector expressing CD14 and *H-2K*^{k} or *H-2K*^{k} alone, respectively. To determine the level of HIV-1 entry efficiency, CD14- or empty vector-transfected $CD4^+$ $CCR5^+$ HeLa cells were challenged with DNase-treated $HIV\text{-}1_{NL4\text{-}3}$. Target cell DNA was isolated at the indicated time and used to detect early reverse transcripts (C), late reverse transcripts (D), 2-LTR circle (E), and the integrated form (F). Data are the means ± SD from duplicate experiments. The levels of the $p24^{gag}$ antigen or HIV-1 DNA in the CD14-transduced cultures were significantly lower than those of the empty vector-transduced cultures ($P < 0.05$, Mann-Whitney U test). Lines with open circles, CD14 vector; lines with filled circles, empty vector.

transfected into $CD4^+$ $CCR5^+$ HeLa cells. More than 70% of cells were confirmed to express the CD14 molecule on the cell surface determined by flow cytometry 2 days after transfection, and then the culture was infected with $HIV\text{-}1_{NL4\text{-}3}$ (X4 virus). The levels of early reverse transcripts, late reverse transcripts, 2-LTR circle, and the integrated form were significantly lower in the CD14-expressing culture than in control culture (Fig. 5C, D, E, and F). However, when $CD4^+$ $CCR5^+$ HeLa cells were infected with $HIV\text{-}1_{NL4\text{-}3}$ and then 2 days later transfected with the CD14-expressing DNA, the HIV-1 release, determined by production of the $p24^{gag}$ antigen in the culture supernatant, was similar in both the CD14-transfected and control cultures (data not shown). These data indicate that CD14 appears to partially inhibit the entry step in HIV-1 replication and provide the HIV CPE-free phenotype.

DISCUSSION

In this study, we established a lentivirus vector system to transduce a cDNA library into human T cells and successfully isolated an anti-CPE gene against HIV-1 infection. A wide variety of expression systems in mammalian cells have been developed, for example, plasmid-based, virus-based, and transposon-based systems. Among them, retrovirus-based and lentivirus-based expression systems can stably transduce genes into human T cells and are, furthermore, efficient enough to screen 10^7 genes. Recently, van Maanen et al. (21) reported the potential use of a lentivirus vector in an expression cloning system. However, the vast majority of cDNA libraries have not been yet constructed on lentivirus vectors. If a cDNA library is efficiently constructed on a lentivirus vector, this vector system will be strongly powerful to isolate genes and accelerate functional genomics. Hence, we used a site-specific recombination system, Gateway, for transferring a cDNA library from a transient expression vector to a lentivirus vector. The major advantage of this transfer system is that we can apply the system to many already established libraries. For instance, a cDNA library amplified in phage can be transferred to a mammalian expression vector including the lentivirus vector without significant loss of its complexity. Many reports have suggested that this technology allows for easy transfer of individual cDNA fragments, and this technology has become a powerful tool for a high-throughput screening system in functional genomics (8, 14, 18, 19). In this study, we used a premade cDNA library in a transient expression vector that cannot be efficiently intro-

FIG. 4. Resistance to HIV-1-induced cell death in CD14-transduced T cells. (A) Flow cytometric analysis of MT-4 cells infected with a CD14- and GFP-expressing lentivirus vector was performed. These cells (GFP^+ *H-2K*$^{k-}$) were mixed with cells infected with a lentivirus vector expressing *H-2K*^{k} alone (GFP^- *H-2K*$^{k+}$) and uninfected cells (GFP^- *H-2K*$^{k-}$) and then challenged with $HIV\text{-}1_{NL4\text{-}3}$. Flow cytometric analysis of the mixed culture is shown before HIV-1 challenge (B), 8 days after HIV-1 challenge (C), and 8 days after mock infection (D). Trypan blue staining (E) and fluorescent microscopic examination (F) were performed 3 days after HIV-1 challenge. A merged image of panels E and F is shown in panel G. Magnification, ×200. Flow cytometric analysis of the HIV-1-challenged culture 10 days after infection (H) or of an uninfected culture (I) was performed by staining with anti-HIV human serum. The results shown are data from one experiment, which is representative of three independent experiments.

duced into T cells. Here, we successfully transferred the cDNA library to a lentivirus vector and found that CD14 can confer resistance to HIV-induced cell death in the transduced cells. This observation suggests that the transferred libraries can still be applied for a functional screening system. Because the vector has also been used to transduce genes into nondividing cells such as neurons (11, 12), muscles (7), and hematopoietic stem cells (4), our lentivirus-based system can be applied to expression cloning systems that use such cells.

However, there is a need to improve our library transfer system. Some degree of loss in library complexity was noted. In our experiment, some of the cDNAs, especially long cDNA fragments, appeared to be lost during two steps: PCR amplification and lentivirus vector production and/or infection. This problem would be solved when the long cDNA fragments are enriched before the BP reaction and a genetic library is directly generated on a donor vector for the LR reaction. We think the latter strategy is useful because, if a library is constructed on a donor vector, the library can be transferred to various expression vector systems, which include not only the lentivirus vector but also other traditional vector systems, by only the LR reaction. As shown in Table 1, we did not observe the loss of library complexity during the LR reaction in our experiment. To overcome the loss of parts of the long cDNA fragments during production of lentivirus vector and/or infection, shorter parent lentiviral vector DNAs should be used in future experiments. In the present study, we used a GFP-expressing lentivirus vector DNA (CSII-EF-MCS-IRES-hrGFP). To obtain long cDNA inserts in the lentiviral vector, there is probably a need to delete some parts of the fragment within the vector DNA, such as IRES-hrGFP.

It was reported that the lentivirus (derived from HIV-1) vector-transduced T cells are less susceptible to wild-type HIV-1 infection than nontransduced T cells (3). The transcripts transduced by the vector appears to compete efficiently for encapsidation, resulting in inhibition of its infectivity, probably because *cis*-acting sequences in the lentivirus vector are responsive to the regulatory protein of wild-type HIV-1. However, the inhibitory effect was completely eliminated in a self-inactivating vector (3). Thus, we used a self-inactivating vector, and we could not actually find any differences in its HIV-1 replication ability between the transduced and nontransduced cells (data not shown).

In the present study, we used a cDNA library as a functional genetic element. In the future, we will be able to choose different genetic libraries, such as ribozyme (8) and peptide libraries (23, 25). The ribozyme library can be efficiently expressed under the control of an RNA polymerase III-dependent promoter (8). When constructing a lentivirus vector containing such a library, the Gateway-based transfer system will be useful. Moreover, since the length of such a library is more homogeneous and shorter than a conventional cDNA library, the lentivirus vector system will be able to more potently deliver a ribozyme library than a cDNA library.

CD14 is known as a coreceptor molecule for lipopolysaccharide (LPS) (24) and is expressed on the surface of myeloid cells via a glycosylphosphatidyl inositol tail. LPS binds to a serum protein, LPS-binding protein (15), and associates with CD14. Subsequently, LPS stimulates Toll-like receptor 4 (13) and activates signaling pathways, mainly the nuclear factor-κB (NF-κB) pathway. HIV-1 also preferentially infects macrophages that express CD14. It is known that macrophages are one of the major target cells for HIV infection, and they behave as cellular reservoirs of virions in HIV-infected patients, probably because the cells are relatively resistant to HIV-induced CPE (5). Although the mechanisms of the low susceptibility of macrophages to HIV-1-induced cell death are poorly understood at present, some explanations may be brought up from the resistance of CD14-transduced cells to HIV-1-induced cell death. One explanation is that overexpression of CD14 can trigger cell survival signals such as NF-κB or induce antiapoptotic genes. Another explanation is that CD14 can reduce the cytotoxicity of HIV-1 infection in T cells through a partial inhibition of HIV-1 replication. In fact, CD14 overexpression resulted in an inhibition of the entry step on HIV-1 replication, as shown in Fig. 5. A determination of the exact mechanisms of CD14 function in HIV-infected cells should enhance our understanding of the cellular events during HIV-induced cell death, which results in immune destruction in HIV-infected individuals.

In conclusion, application of the Gateway system to a genetic library transfer system will allow the use of the lentivirus vector system as a powerful tool for the study of functional genomics of mammalian cells.

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Intrinsic and spontaneous neurogenesis in the postnatal slice culture of rat hippocampus

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Keywords: adult neurogenesis, calbindin, dentate gyrus, enhanced green fluorescent protein, virus

Abstract

Organotypic slice culture preserves the morphological and physiological features of the hippocampus of live animals for a certain time. The hippocampus is one of exceptional regions where neurons are generated intrinsically and spontaneously throughout postnatal life. We investigated the possibility that neurons are generated continuously at the dentate granule cell layer (GCL) in slice culture of the rat hippocampus. Using 5-bromodeoxyuridine (BrdU) labelling and retrovirus vector transduction methods, the phenotypes of the newly generated cells were identified immunohistochemically. At 4 weeks after BrdU exposure, BrdU-labelled cells were found in the GCL and were immunoreactive with a neuronal marker, anti-NeuN. There were fibrils immunoreactive with anti-glial fibrillary acidic protein (GFAP), an astrocyte marker, in the layer covering the GCL and occasionally encapsulated BrdU-labelled nuclei. When the newly divided cells were marked with the enhanced green fluorescent protein (EGFP) using a retrovirus vector, these cells had proliferative abilities throughout the following 4-week cultivation period. Four weeks after the inoculation, the EGFP-expressing cells consisted of various phenotypes of both early and late stages of differentiation; some were NeuN-positive cells with appearances of neurons in the GCL and some were immunoreactive with anti-Tuj1, a marker of immature neurons. Some EGFP-expressing cells were immunoreactive with anti-GFAP or anti-nestin, a marker of neural progenitors. The present study suggests that slice cultures intrinsically retain spontaneous neurogenic abilities for their cultivation period. The combination of slice culture and retrovirus transduction methods enable the newly divided cells to be followed up for a long period.

Introduction

In the mammalian central nervous system, the hippocampus is one of regions where new neurons are generated intrinsically and spontaneously throughout the postnatal life of animals (Altman & Das, 1965; Cameron *et al.*, 1993; Gould *et al.*, 1999b; Kornack & Rakic, 1999) including humans (Eriksson *et al.*, 1998). These cells acquire neuron-like appearances with axons and dendrites (van Praag *et al.*, 2002), neuron-specific marker proteins such as NeuN and Tuj1 (Cameron *et al.*, 1993; Eriksson *et al.*, 1998; Kornack & Rakic, 1999; van Praag *et al.*, 2002) and neuron-like membrane properties such as action potentials and synaptic potentials (van Praag *et al.*, 2002) during the course of maturation. Moreover, these cells migrate to be integrated in the dentate granule cell layer (GCL), express calbindin D28K, a GCL cell marker protein (Kuhn *et al.*, 1996; Eriksson *et al.*, 1998) and project axons into the CA3 region (Hastings & Gould, 1999; Markakis & Gage, 1999). Neural stem cells derived from the adult hippocampus differentiate into neuron-like cells and form synaptic networks *in vitro* (Song *et al.*, 2002). These morphological and physiological features strongly suggest that the new neurons would be incorporated in the hippocampal local circuitry and that they would be involved in hippocampus-dependent memory formation (Shors *et al.*, 2001; Kempermann, 2002; van Praag *et al.*, 2002) and brain repair (Gould & Tanapat, 1997; Liu *et al.*, 1998; Kuhn *et al.*, 2001).

Organotypic cultures of hippocampal slices would provide an alternative model to hippocampus *in vivo* (Gähwiler *et al.*, 1997). As all types of neurons and glia are preserved with their specific morphologies and localizations in hippocampal slice cultures, the main network organization is very similar to that of living animals (Gähwiler, 1984; Zimmer & Gähwiler, 1984; Dailey *et al.*, 1994). However, a few rearrangements have been observed probably as a result of afferent deprivation (Robain *et al.*, 1994; Gutierrez & Heinemann, 1999). Neurons in slice culture maintained their physiological membrane properties and synaptic transmissions as well as several forms of short- and long-term plasticity (Gähwiler *et al.*, 1997). Therefore, slice cultures have been used widely in studies on physiology, pharmacology, morphology and the plasticity of the hippocampus.

It is plausible that hippocampal slice cultures have potencies to generate new neurons *in vitro* as suggested by the observations that neural progenitor-like cells are found in the slice culture (Miyaguchi, 1997) and transplanted ES cells differentiate into neurons (Benninger

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et al., 2003). The presence of neurogenic abilities has recently been reported in hippocampal slice cultures by 5-bromodeoxyuridine (BrdU) labelling methods (Raineteau *et al.*, 2004). In the present study the newly generated cells were also identified by retrovirus vector transduction methods. We found that hippocampal slice cultures retain endogenous neural progenitors in the dentate gyrus throughout the cultivation period and maintain *per se* the potential to differentiate spontaneously into neurons. We followed up the newly generated cells for a long period and found that some newly generated neurons are indeed incorporated into the normal architecture of GCL with the expression of one of the GCL cell markers, calbindin D28K.

Materials and methods

Slice culture

Hippocampal organotypic cultures were prepared according to the standard interface method (Stoppini *et al.*, 1991; Sakaguchi *et al.*, 1994). Hippocampal slices were prepared from postnatal day 7 Wistar rats (Nihon SLC Co., Japan). After decapitation, the brain was removed and transversely sliced at the hippocampus into a 350 μm thickness on a McIlwain tissue chopper (The Mickle Laboratory Engineering, UK). The isolated hippocampal slices were transferred onto a porous translucent membrane (Millicell-CM: PICM03050, Millipore, Billerica, MA, USA) and maintained in culture at 34 °C for several weeks in the interface between culture medium and a 5% CO_2 atmosphere. The culture medium in this experiment was a mixture of 50% commercial medium (OPTI-MEM, Invitrogen, Carlsbad, CA, USA), 25% heat inactivated horse serum (Invitrogen) and 25% Hank's balanced salt solution (Invitrogen) supplemented with D-glucose (5 g/L), penicillin (100 units/mL; Invitrogen) and streptomycin (100 μg/mL; Invitrogen), and was changed twice a week. Slices were also prepared from an adult female Wistar rat, as a control. The animal was deeply anaesthetized with diethyl ether then decapitated. The brain was removed and hippocampal slices (100 μm thickness) were prepared. All of the animal treatments used here were performed in accordance with the guidelines laid down by the Japan Neuroscience Society and NIH.

To label the immediately post-mitotic cells, slices at 13–14 days *in vitro* (DIV) were incubated for 3 days with culture medium containing 10 μM 5-bromodeoxyuridine (BrdU, Roche Diagnostics, Indianapolis, IN, USA) and then to normal medium. One to four weeks after BrdU treatment, the slices were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 1 h at 4 °C.

Retrovirus vector transduction

A murine leukaemia virus-based vector, SRαLEGFP (An *et al.*, 1999), was derived from SRα*Lthy* (An *et al.*, 1997) by replacing the murine thy1.2 gene with enhanced green fluorescent protein (EGFP). Vector stocks were generated by calcium phosphate-mediated transfection of HEK 293T cells. HEK 293T cells were cultured in Dulbecco's modified Eagle medium with 10% calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator and transfected the following day with the envelope plasmid pMD.G (Zufferey *et al.*, 1997), the packaging plasmid pSVψ-E-murine leukaemia virus (Landau & Littman, 1992) and the transfer vector plasmid SRαLEGFP. At 24 h post-transfection, the medium was replaced with serum-free medium (OPTI-MEM; Invitrogen). At 48 h post-transfection, the virus-containing supernatant was collected, centrifuged and passed through a 0.45 μm filter. The virus vectors were further concentrated by centrifugation at 6000 *g* for 16 h at 4 °C. The pellet was resuspended in serum-free medium and kept in liquid nitrogen until use. Stocks of the vector were titrated by infecting NIH3T3 cells and analysing for EGFP expression by flow cytometry. The titre of vectors was 5×10^5 infectious units/mL.

Slices at 14 DIV were inoculated with the retrovirus vector encoding EGFP in the suprapyramidal region of the GCL. Then, 2–4 weeks later, the slices were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 1 h at 4 °C and used for immunohistochemical studies.

Zinc histofluorescence

The distribution of axons (mossy fibres; MFs) and their terminals in GCL neurons was investigated using a membrane-permeable zinc-sensitive fluorescent indicator, *N*-(6-methoxy-8-quinolyl)-*p*-toluenesulphonamide (TSQ, Molecular Probes, Eugene, OR, USA). TSQ (5 mg) was dissolved in 333 μL dimethyl sulphoxide (DMSO) containing 20% pluronic acid (Dojindo Laboratories, Kumamoto, Japan) and stored at 4 °C. A TSQ solution (1 : 250) was freshly prepared by adding Ca^{2+}-free Tyrode's solution, which reduced the release probability and thereby prevented translocation of the zinc from presynaptic terminals. The vital or freshly frozen slices at 2–6 weeks *in vitro* (WIV) were immersed in the TSQ solution for 30 min at 34 °C and briefly rinsed in Tyrode's solution containing EDTA disodium-calcium salt (Ca-EDTA, 10 mM) to chelate extracellular free zinc (Varea *et al.*, 2001). The slices were finally viewed by using a conventional epifluorescence microscope (BX51, Olympus, Tokyo, Japan) equipped with a WU excitation/detection filter and recorded using a digital camera system (PDMC-II, Polaroid, Cambridge, MA, USA).

Immunohistochemistry

The slices were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer at 4 °C for 1 h, then washed in PBS. For BrdU detection, the slices were incubated in 2 N HCl at 37 °C for 1 h to denature DNA, neutralized twice with 0.1 M borate buffer (pH 8.5) for 30 min and washed twice in PBS. After peeling the slices off from the membrane, all the subsequent procedures were carried out on free-floating slices using a rotator. The slices were blocked in PBS with 5% normal goat serum and 0.3% Triton X-100 at 4 °C overnight and reacted with the following primary antibodies in phosphate-buffered saline (PBS) with 5% normal goat serum and 0.3% Triton X-100 at 4 °C for 24 h: mouse monoclonal anti-NeuN (1 : 1000; Chemicon, Temecula, CA, USA), mouse monoclonal anti-βIII tubulin (Tuj1; 1 : 1000; Promega, Madison, WI, USA), mouse monoclonal anti-MAP2 (1 : 1000; Sigma-Aldrich, St Louis, MO, USA), mouse monoclonal anti-nestin (1 : 1000; Chemicon), guinea pig polyclonal anti-GFAP (1 : 1000; Advanced Immunochemical, Long Beach, CA, USA), rabbit polyclonal anti-GFAP (1 : 1000; Promega), rat monoclonal anti-BrdU [Clone:BU1/75 (ICR1); 1 : 100; Oxford Biotechnology, UK] or rabbit polyclonal anti-EGFP produced by Tamamaki *et al.* (2000) (1 : 500; a generous gift from Dr Y. Yanagawa, Gunma University, Japan). The preparations were then washed three times in PBS with 0.1% Triton X-100 and incubated at room temperature for 5–6 h with fluorescent dye conjugated secondary antibodies (each, 1 : 200) from different species: Alexa Fluor 546-conjugated anti-mouse IgG; Alexa Fluor 488-conjugated anti-guinea pig IgG; Alexa Fluor 488-, 546- or 633-conjugated anti-rat IgG; and Oregon Green 488-conjugated anti-rabbit IgG (all purchased from Molecular Probes). To ensure that our labelling patterns were not the conse-