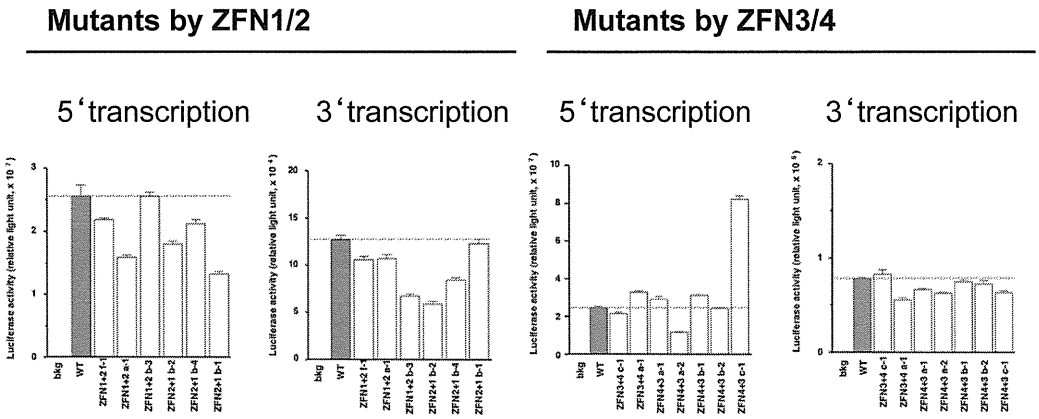
図 1 . Inhibition of cell growth by ZFN in HTLV-1-transformed T cell line C/M8166

	Cloning efficiency				
	Expt 1	Expt 2	Expt 3	Expt 4	Mean
ZFN1/2 pair					
ZFN1+1	1.3	1.0	n.t.	n.t.	1.15
ZFN1+2	0.0	0.2	0.2	0.1	0.14
ZFN2+1	0.0	0.0	0.1	0.1	0.04
ZFN2+2	3.1	4.4	n.t.	n.t.	3.75
ZFN3/4 pair					
ZFN3+3	5.4	3.3	n.t.	n.t.	4.38
ZFN3+4	0.4	0.2	n.t.	n.t.	0.31
ZFN4+3	0.4	0.2	0.5	n.t.	0.36
ZFN4+4	8.8	10.0	n.t.	n.t.	9.38

図 2 . Inhibition of cell growth by ZFN in HTLV-1-positive ATL-derived T cell line S1T

	Cloning efficiency				
	Expt 1	Expt 2	Expt 3	Expt 4	Mean
ZFN1/2 pair					
ZFN1+1	0.52	0.50	n.t.	n.t.	0.51
ZFN1+2	0.11	0.15	n.t.	n.t.	0.13
ZFN2+1	0.11	0.10	n.t.	n.t.	0.11
ZFN2+2	0.63	0.45	n.t.	n.t.	0.54
ZFN3/4 pair					
ZFN3+3	0.42	0.41	0.22	0.11	0.41
ZFN3+4	0.33	0.38	0.10	0.07	0.35
ZFN4+3	0.18	0.13	n.t.	n.t.	0.15
ZFN4+4	0.55	0.52	n.t.	n.t.	0.54

図 3 . Effect of mutations on LTR-driven transcription



The Tax-augmented transcription of the positive strand was reduced by most of the mutations introduced by ZFN1/2 (4/6 clones, Fig. 3D). Similarly, the negative strand transcription was also reduced by ZFN1/2-induced mutations (5/6 clones). On the other hand, increased positive strand transcription was observed in 4/7 LTR mutants recovered from ZFN3/4-transduced cells. By contrast, many of the LTR mutants showed modestly reduced negative strand transcription.

III. 平成２３-２５年度　業績一覧

研究成果の刊行に関する一覧表

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Ⅳ. 平成２３−２５年度　刊行物別冊（抜粋）

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ORIGINAL ARTICLE

A novel therapeutic molecule against HTLV-1 infection targeting provirus

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Human T-cell leukemia virus type 1 (HTLV-1), which causes adult T-cell leukemia (ATL) in humans, establishes a life-long latent infection. Current therapies are not very effective against HTLV-1-associated disorders. A novel therapeutic approach may help to combat HTLV-1 infection. A molecular therapy that targets the proviral genome is favorable because the therapeutic effect occurs specifically in HTLV-1-infected cells, regardless of whether they express viral genes. In this proof-of-concept study, we developed a therapeutic molecule based on zinc finger nuclease (ZFN) to achieve this goal. We designed a ZFN that specifically recognized conserved region of HTLV-1 long terminal repeat (LTR) and introduced it into various HTLV-1-positive human T-cell lines, including HTLV-1-transformed and ATL-derived cell lines. The ZFN disrupted the promoter function of HTLV-1 LTR and specifically killed HTLV-1-infected cells. We also showed a potential approach of this therapeutic molecule to remove the proviral genome from HTLV-1-infected cells, something that has not been possible before. The therapeutic effect of ZFN was confirmed in an *in vivo* model of ATL. This strategy may form the basis of a therapy that can eradicate HTLV-1 infection. Similar approaches can be used to target other malignancy-associated viruses.

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Keywords: Human T-cell leukemia virus type 1; zinc finger nuclease; molecular therapy; adult T-cell leukemia

INTRODUCTION

Human T-cell leukemia virus type I (HTLV-1) is a retrovirus that causes adult T-cell leukemia (ATL) and the neurological disorder, HTLV-1-associated myelopathy, in humans.[1–3] HTLV-1 establishes a life-long latent infection because the viral genome is integrated into the host cell DNA. HTLV-1 undergoes the lytic phase of the viral life cycle in some infected cells, whereas most of the other cells remain latently infected. Once the viral genes are expressed, the host immune system can remove infected cells; however, it is difficult to eradicate HTLV-1-positive cells completely from infected individuals because latently infected cells do not actively transcribe the viral genes. No cell surface markers have been identified that distinguish HTLV-1-infected cells from uninfected cells. No effective vaccine has yet been developed, and HTLV-1-associated disorders respond poorly to current therapies.[4,5] Thus, a molecular therapy is urgently needed. Considering the phenomenon of viral latency, it is reasonable to target the provirus. Here, we used artificial endonuclease technology to achieve this goal.

Zinc finger nuclease (ZFN), a synthetic endonuclease, introduces a double strand break (DSB) into its cognate DNA site, thereby activating DNA damage-induced apoptosis.[6–8] Therefore, we hypothesized that targeting the HTLV-1 provirus with ZFN should kill HTLV-1-infected cells by DSB-induced apoptosis, resulting in the elimination of provirus-positive cells from the virus-infected individuals (Supplementary information S1). Some of the DNA strand break sites are repaired by DNA damage-response mechanisms such as non-homologous end joining, which ligates the broken ends of the chromosomal DNA. When this occurs, the original DNA sequence is disrupted. The integrated retroviral genome has two identical copies of a sequence known as the viral long terminal repeat (LTR). Targeting the LTR is advantageous because the number of therapeutic targets per provirus is doubled (no other part of the viral genome has two copies). The LTR acts as a viral promoter; therefore, even if cell death is not induced, the proviral DNA should be irreversibly damaged, thereby reducing expression of the viral genes and subsequent pathogenicity. Furthermore, ZFN removes a DNA fragment from the chromosome.[1] This is possible when two DSBs are introduced into the chromosomal DNA proximal to each other. The DSB repair system ligates the DNA ends and removes the short DNA fragment generated by the two DSBs (a process termed as targeted deletion activity; Supplementary information S1). Thus, it should be possible to delete the proviral gene if a repetitive element, such as the LTR, which is positioned at either ends of the provirus, is targeted by ZFN. Therefore, we conducted a proof-of-concept study in which we used ZFN to damage the HTLV-1 provirus in infected cells and inhibit cell proliferation of virus-infected cells. It was possible to design a ZFN molecule with the required properties because the endonuclease has high-substrate specificity, and the HTLV-1 LTR has limited sequence diversity compared with the LTR of human immunodeficiency virus.

MATERIALS AND METHODS

Cells and transfection

All T-cell lines were maintained in RPMI 1640 (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Japan Bioserum, Tokyo, Japan),

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penicillin and streptomycin (Invitrogen, Tokyo, Japan). Dulbecco's modified eagle medium (Sigma) was used to culture the 293T cells. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were transfected using Lipofectamine 2000 (Invitrogen) or FuGENE6 transfection reagents (Roche, Tokyo, Japan). The S1T and ED cell lines were generous gifts from Drs Baba (Kagoshima University) and Matsuoka (Kyoto University).

Plasmids and the murine leukemia virus *vector*

ZFN genes were purchased from Sigma. The retroviral plasmids pQc, pCMMP and pMX were constructed using standard molecular cloning techniques. The Tax-expressing plasmid, pCGtax, and pHTLV LTR-luciferase are kindly provided by Dr Watanabe (Tokyo University). The production of, and infection by, murine leukemia virus (MLV) vectors has been described previously.[9–11]

Cell imaging

Cells were imaged by confocal fluorescence microscopy (LSM510 Meta ×40 NA 1.4 lens, Carl Zeiss MicroImaging Inc., Tokyo, Japan) or fluorecence microscopy (Olympus IX70, Tokyo, Japan).

Table 1. Targeted deletion activity of ZFN.

Reporter	*Effector*	*No. of colonies*[a]	*No. of white colonies*	*% of white colonies*
ZFN1 + 2-specific deletion reporter	None	6562	2	0
	ZFN1	3050	9	0.3
	ZFN2	2268	6	0.3
	ZFN1 + ZFN2	905	75	8.3
ZFN3 + 4-specific deletion reporter	None	5379	1	0
	ZFN3	3959	13	0.3
	ZFN4	3206	14	0.4
	ZFN3 + ZFN4	1722	38	2.2

Abbreviation: ZFN, zinc finger nuclease. [a]Number of bacterial colonies emerged on a 10-cm Luria-Bertani agar plate containing x-Gal.

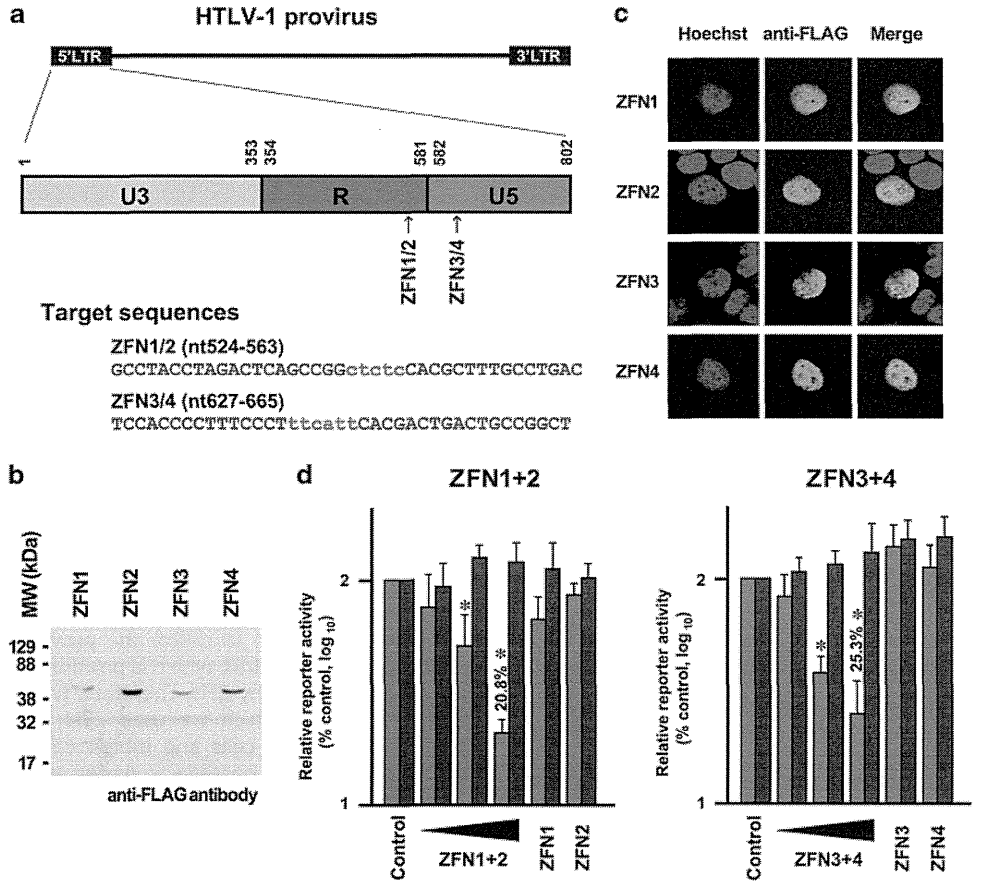

Figure 1. Characterization of ZFN function using reporter constructs. (**a**) Structure of the HTLV-1 provirus and the targets of ZFN1/2 and ZFN3/4. The nucleotide number coordinates are derived from GenBank accession number D13784. The uppercase letters in blue represent the ZFN recognition sequence and the lowercase letters in red represent the linker into which DSBs are introduced. (**b**) Confirmation of ZFN expression in transfected 293T cells by western blotting of cell lysates with an anti-FLAG antibody. (**c**) Immunofluorescence assay showing the distribution of ZFNs in 293T cells transfected with each expression plasmid. ZFNs detected with an anti-FLAG antibody are green and the Hoechst 33258-stained nuclei are blue. Magnification is ×400. (**d**) Transient transfection assay to examine the effect of ZFN on Tax-augmented LTR transcription. The ZFN expression vectors (20, 100 or 500 ng; containing each subunit alone or an equal amount of each subunit (1/2 or 3/4), Tax (100 ng) and a reporter plasmid encoding the LTR-luciferase cassette (200 ng) were cotransfected into 293T cells, and the luciferase signals were detected at 2 days post transfection (red). The expression vector containing GFP was used as the control. The total amount of transfected plasmid was adjusted using a plasmid-expressing GFP. A CMV-driven luciferase expression vector (50 ng) was used for comparison (blue). The average and standard deviation of the luciferase signals relative to that of the control is shown from four independent experiments. Statistically significant differences between each plasmid and the corresponding CMV control were analyzed using a two-tailed Student's *t*-test (asterisk denotes $P<0.01$).

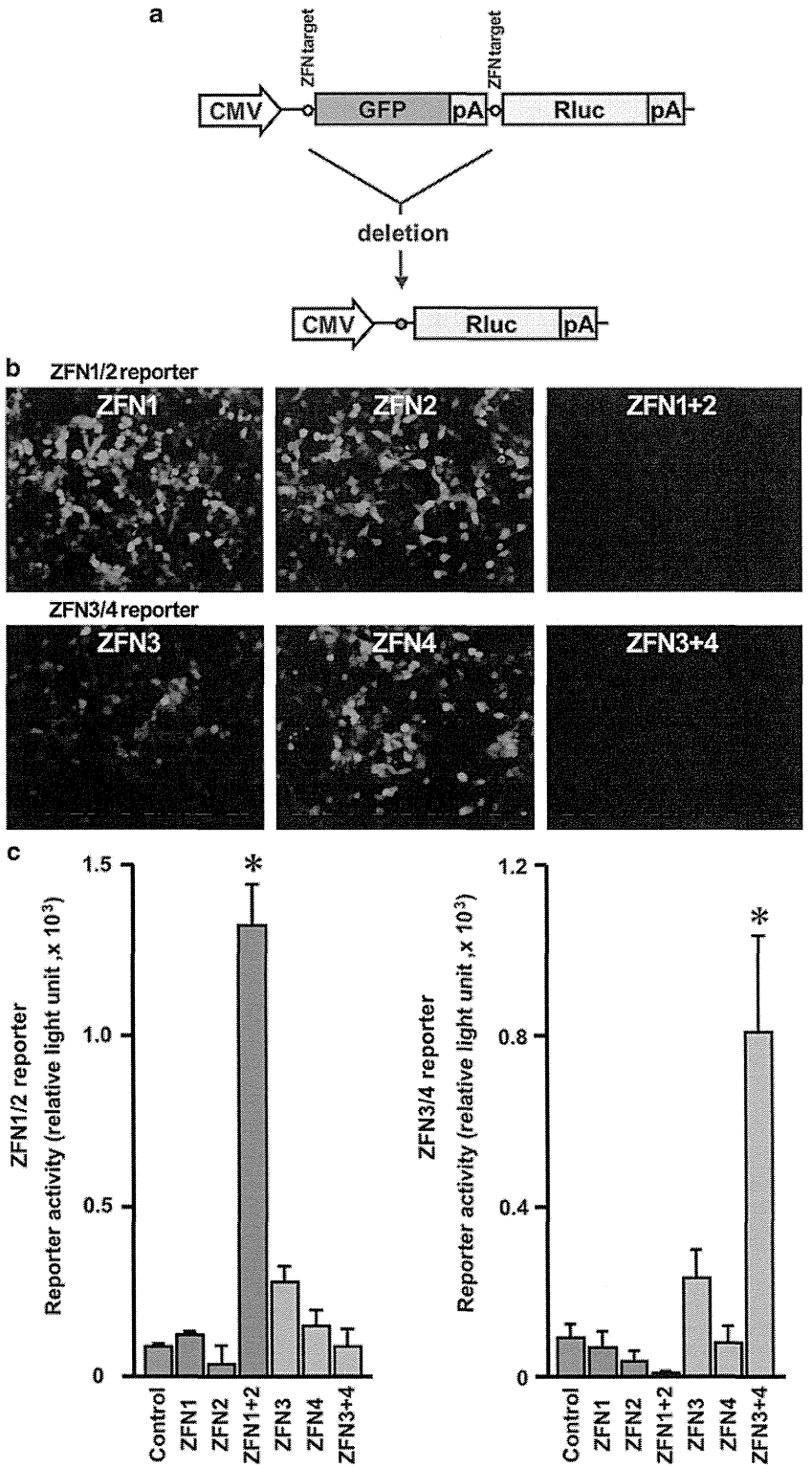

Figure 2. *Detection of ZFN-targeted deletion activity.* (**a**) Schematic representation of the reporter plasmid used to detect the ability of ZFN to remove a DNA fragment flanked by two ZFN recognition sites. The expression of renilla luciferase (Rluc) is driven by the CMV promoter when the GFP expression cassette is removed by ZFN. The ZFN target sites are indicated by circles. pA is polyadenylation signal. (**b**) The ZFN1/2 or 3/4 reporter plasmids were transfected into 293 T cells in the presence of ZFN expression vectors as indicated in each panel. The cells were observed under a fluorescence microscope at a low magnification (× 100) at 2 days post transfection. (**c**) Transient transfection assay to assess the targeted deletion activity of ZFN. The ZFN expression vectors (500 ng; containing each subunit alone or equal amounts of each ZFN subunit) and the reporter plasmid (50 ng) were cotransfected into 293 T cells and the luciferase signals were detected at 2 days post transfection. A GFP expression vector was used as the control. Representative data from four independent experiments, each performed in triplicate, are shown. Significant differences between the ZFN pairs and the subunits alone were analyzed using a two-tailed Student's *t*-test (asterisk denotes $P<0.001$).

Table 2. Cloning efficiency of HTLV-1-infected cells transduced with ZFN.

Cells	*ZFN*[a]	*Cloning efficiency (%, mean ± s.d.)*	*Statistical significance*
M8166	ZFN1 + ZFN2	0.14 ± 0.1 (*N* = 4)	*P* = 0.0013, 26.9-fold, 8 vs 4
	ZFN2 + ZFN1	0.04 ± 0.05 (*N* = 4)	
	ZFN1 + ZFN1	1.15 ± 0.15 (*N* = 2)	
	ZFN2 + ZFN2	3.75 ± 0.88 (*N* = 2)	
	ZFN3 + ZFN4	0.31 ± 0.15 (*N* = 2)	*P* = 0.0018, 20.0-fold, 4 vs 5
	ZFN4 + ZFN3	0.36 ± 0.14 (*N* = 2)	
	ZFN3 + ZFN3	4.38 ± 1.47 (*N* = 3)	
	ZFN4 + ZFN4	9.38 ± 0.88 (*N* = 2)	
S1T	ZFN1 + ZFN2	0.13 ± 0.02 (*N* = 2)	*P* < 0.0001, 4.4-fold, 4 vs 4
	ZFN2 + ZFN1	0.11 ± 0.01 (*N* = 2)	
	ZFN1 + ZFN1	0.51 ± 0.01 (*N* = 2)	
	ZFN2 + ZFN2	0.54 ± 0.13 (*N* = 2)	
	ZFN3 + ZFN4	0.35 ± 0.03 (*N* = 4)	*P* = 0.08, 1.9-fold, 6 vs 6
	ZFN4 + ZFN3	0.15 ± 0.04 (*N* = 2)	
	ZFN3 + ZFN3	0.41 ± 0.01 (*N* = 4)	
	ZFN4 + ZFN4	0.54 ± 0.02 (*N* = 2)	

Abbreviations: HTLV-1, human T-cell leukemia virus type 1; ZFN, zinc finger nuclease. [a]The order of transduction into cells is indicated.

Table 3. Detection of ZFN-mediated site-directed mutagenesis in S1T cells.

ZFN[a]	*No. of clones examined*	*No. of clones with site-specific mutation*
ZFN1 + ZFN2	9	3
ZFN2 + ZFN1	7	3
ZFN1 + ZFN1	1	0
ZFN2 + ZFN2	1	0
ZFN3 + ZFN4	12	4
ZFN4 + ZFN3	6	5
ZFN3 + ZFN3	3	0
ZFN4 + ZFN4	2	0

Abbreviation: ZFN, zinc finger nuclease. [a]The order of transduction into cells is indicated.

Reporter assay

Luciferase activity was measured 48 h after transfection or infection using a Dual-Glo or Renilla-Glo Luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Chemiluminescence was detected with a Veritas luminometer (Promega).

Immunoblotting

Western blotting and immunoprecipitation were performed as previously described[12] using an anti-FLAG antibody (F7425, Sigma) and Envision Dual Link System-HRP (Dako, Glostrup, Denmark). Chemiluminescence was generated using Lumilight (Roche) or Lumigen (GE Healthcare, Tokyo, Japan), and the signals were detected using an LAS-3000 mini Lumino-Image analyzer.

Cell proliferation assay

C8166 and S1T cells were transduced with ZFN genes inserted into the MLV vector and bulk-selected in medium containing 1 μg/ml puromycin. The cells were then transduced with a second *ZFN* gene in the MLV vector, seeded into 96-well plates (5 or 100 cells/well), and selected in medium containing 500 μg/ml G418 at 3 days post infection. The number of wells containing viable cells was counted at 3–4 weeks post selection. Cellular DNA was isolated using the Wizard DNA Purification Kit (Promega), and the proviral DNA sequence was analyzed by sequencing after PCR amplification of the target regions.

Mouse study

Balb/c nude Rag-2/Jak3 double-deficient (nude R/J) mice were established by crossing Balb/c nude mice and Balb/c Rag-2/jak3 double-deficient mice.[13] Nude R/J mice were inoculated subcutaneously in the right and left flanks with 2×10^5 $ED^{ZFN2/ZFN1}$ and $ED^{ZFN2/ZFN2}$ cells, respectively. Tumor growth was monitored at 6 weeks post inoculation by weekly measurement of the maximal and minimal diameter using calipers. Tumor size was estimated using the formula: tumor size (mm^3) = length (mm) × $width^2$ (mm) × 0.4.[14,15] The mice were housed and monitored in the animal research facility according to institutional guidelines. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee at Kumamoto University (B23-201).

Statistical analysis

Experimental results were analyzed by Student's *t*-test, Person's exact test or Paired Wilcoxon Rank-Sum test, as appropriate. $P < 0.05$ were considered statistically significant.

RESULTS

We synthesized two pairs of ZFNs that targeted the HTLV-1 LTR, which are: ZFN1/2, which recognizes the R region (524–563 bp, according to the GenBank accession number D13784 coordinate) and ZFN3/4, which recognizes the U5 region (627–665 bp) (Figure 1a). According to our own data and the NCBI database, these sequences are conserved in 77.8% (14/18 clones) of independent HTLV-1 clones isolated in Asia, Europe, and North and South America. Sequence analysis indicated that the human genome does not contain sequences that are identical to these ZFN targets. ZFN was tagged at the N-terminus with a 3× FLAG epitope and ZFN expression was verified by western blotting of 293 T cells transfected with the ZFN expression vectors (Figure 1b). As the ZFNs were fused to a nuclear localization signal, they were distributed predominantly in the nucleus (Figure 1c).

Next, ZFN activity was examined in human cells. First, we assessed whether ZFN inhibited LTR-driven gene expression. For this purpose, ZFN expression vectors were transfected into 293 T cells along with a Tax expression vector and a reporter plasmid encoding an HTLV-1 LTR-luciferase cassette. Luciferase activity was markedly reduced upon coexpression of functional ZFN pairs (Figure 1d). This effect was dependent upon the amount of plasmid transfected. By contrast, ZFN expression did not affect the transcriptional activity of the cytomegalovirus (CMV) promoter (Figure 1d). Second, two deletion reporter plasmids were constructed to test the targeted deletion activity. One deletion reporter harbored a CMV promoter and green fluorescent protein (GFP) and renilla luciferase genes (Figure 2a). The *GFP* gene was sandwiched by the cognate ZFN site (Figure 2a). The renilla luciferase was not expressed unless the GFP cassette was removed. The results of a cotransfection experiment in 293 T cells showed that GFP fluorescence decreased substantially in the presence of functional ZFN subunits (Figure 2b), whereas a marked increase in renilla luciferase activity was detected upon coexpression of functional ZFN subunits (Figure 2c).

To confirm this finding, we constructed another deletion reporter containing a bacterial LacZ-alpha expression cassette flanked by ZFN cognate sites and cotransfected it into 293 T cells with the ZFN expression plasmids. *Escherichia coli* were then transformed with DNA recovered from the transfected 293 T cells. Deletion of the LacZ-alpha expression cassette should result in white bacterial colonies on agar plates containing X-Gal. As expected, the number of white colonies increased substantially when the ZFN pairs were expressed in 293 T cells (Table 1). Interestingly, expression of the ZFN pairs caused a reduction in the number of colonies, suggesting that DSBs were efficiently introduced into the reporter plasmid, but not all of the DSB loci were ligated by the DSB repair system in 293 T

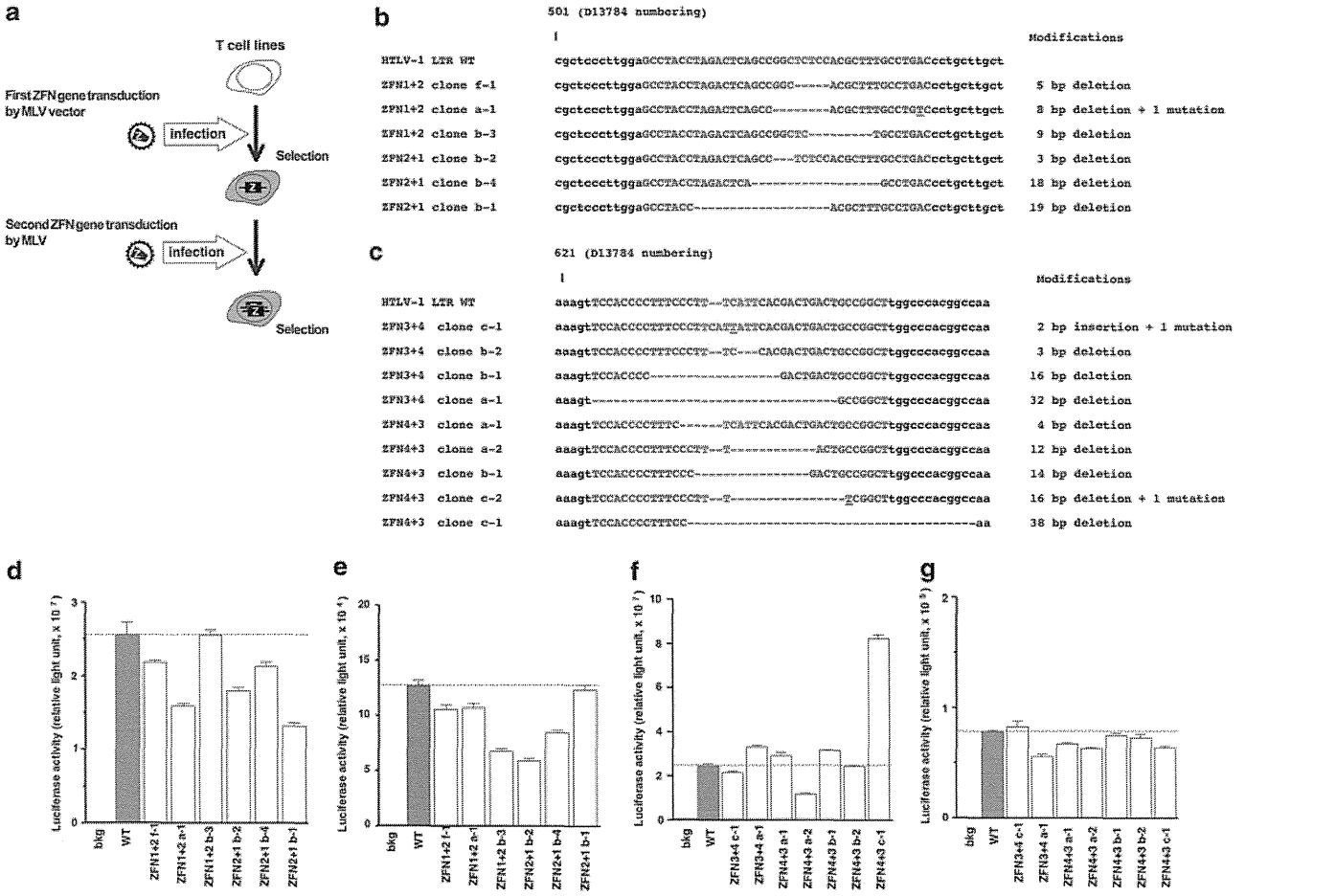

Figure 3. Effect of ZFN-mediated site-directed mutagenesis of the HTLV-1 LTR. (**a**) Experimental procedure used to assess the effect(s) of ZFN expression in HTLV-1-infected cells. (**b** and **c**) Sequence of the LTR (spanning the ZFN target sites) recovered from S1T cells transduced with ZFN1/ZFN2 (**b**) or ZFN3/ZFN4 (**c**). The nucleotide number coordinates are derived from GenBank accession number D13784. Blue letters represent the ZFN recognition sequence and red letters represent the DSB induction sites as in Figure 1a. Dashes represent deletions and underlining represents substitutions. (**d**–**g**) Reporter assay to assess the promoter activity of the LTR mutants shown in (**b**) and (**c**). The Tax expression vector (100 ng) and the reporter plasmid carrying the LTR mutants (200 ng) were cotransfected into 293 T cells and the luciferase signals were detected at 2 days post transfection. The transcriptional activity of the positive (**d** and **f**) and negative strand (**e** and **g**) is indicated. Data are representative of four independent experiments. The dashed line indicates the wild-type level.

cells. A small number of white colonies were observed, presumably because ZFN-independent deletion of LacZ from the reporter plasmid occurs spontaneously and with low frequency. Taken together, these data suggest that ZFNs inhibit the function of the LTR promoter and remove DNA flanked by the ZFN cognate sites. We assume that the inhibition of LTR promoter function (Figure 1d) was caused by physical damage to the LTR.

We next examined the biological effect(s) of ZFNs in HTLV-1-transformed and ATL-derived CD4-positive human T-cell lines, harboring the HTLV-1 provirus. HTLV-1 immortalizes human CD4-positive T cells, and the proliferation of HTLV-1-transformed cells is dependent on HTLV-1 gene expression. If ZFN inhibits the promoter function of the LTR, then ZFN expression should inhibit the proliferation of HTLV-1-transformed cell lines. In addition, HTLV-1-positive cells should undergo DSB-triggered apoptosis upon ZFN expression. We confirmed that the ZFN target sequence in the LTR was conserved in all the cell lines used in this study. Initially, we transduced a subunit of ZFN into C8166 cells[16] using a MLV vector. A second ZFN subunit was then transduced into the cells using a MLV vector carrying a distinct selection marker, and cell proliferation was examined by measuring the cell-cloning efficiency (Figure 3a). For the control cells, the same ZFN subunit that was transduced initially was introduced again (referred to as a "non-functional pair"). When the functional ZFN1/2 and ZFN3/4 pairs were expressed, the cloning efficiency of C8166 cells was significantly reduced by 26.9- and 20.0-fold, respectively, compared with that of the control ($P=0.001$ and $P=0.002$, respectively; Student's *t*-test, Table 2). Next, we examined the ATL cell line S1T, which proliferates in an interleukin-2-independent manner.[17] Under the same experimental conditions, the cloning efficiency of S1T cells was reduced by 4.4- and 1.9-fold compared with the control when functional ZFN1/2 and ZFN3/4 pairs, respectively, were expressed (Table 2). The reduction in the cell-cloning efficiency mediated by ZFN1/2 (but not ZFN3/4) was statistically significant ($P<0.001$; Student's *t*-test). Proliferative inhibition of HTLV-1-transformed and ATL-derived cell lines was observed when cell growth was assessed in bulk culture using metabolic measurements (data not shown). We observed similar results in HTLV-1-transformed MT-2 and MT-4 cells, and the ATL-derived cell lines, ED and TL-Oml (data not shown). Furthermore, these findings were not observed in four HTLV-1-negative CD4-positive T-cell lines, including CEM, MOLT-4, Jurkat and SUP-T1 (data not shown). These data suggest that ZFN inhibits the proliferation of HTLV-1-infected cells, specifically. We also noted that the cell-cloning efficiency was reduced to a greater extent in