Genes: Structure and Regulation:

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Artificial Zinc Finger Fusions Targeting Sp1-binding Sites and the *trans*-Activator-responsive Element Potently Repress Transcription and Replication of HIV-1*

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Tat activates transcription by interacting with Sp1, NF-κB, positive transcription elongation factor b, and *trans*-activator-responsive element (TAR). Tat and Sp1 play major roles in transcription by protein-protein interactions at human immunodeficiency virus, type 1 (HIV-1) long terminal repeat. Sp1 activates transcription by interacting with cyclin T1 in the absence of Tat. To disrupt the transcription activation by Tat and Sp1, we fused Sp1-inhibiting polypeptides, zinc finger polypeptide, and the TAR-binding mutant Tat (TatdMt) together. A designed or natural zinc finger and Tat mutant fusion was used to target the fusion to the key regulatory sites (GC box and TAR) on the long terminal repeat and nascent short transcripts to disrupt the molecular interaction that normally result in robust transcription. The designed zinc finger and TatdMt fusions were targeted to the TAR, and they potently repressed both transcription and replication of HIV-1. The Sp1-yinhibiting POZ domain, TatdMt, and zinc fingers are key functional domains important in repression of transcription and replication. The designed artificial zinc fingers were targeted to the high affinity Sp1-binding site, and by being fused with TatdMt and POZ domain, they strongly block both Sp1-cyclin T1-dependent transcription and Tat-dependent transcription, even in the presence of excess expressed Tat.

More than 35 million people worldwide are infected with HIV,[1] and most of them will develop AIDS (Ref. 1 and references therein). A mixture of reverse transcriptase and protease inhibitors can reduce the amount of HIV in the blood significantly. However, the severe side effects of these drugs, expensive cost, and viral mutation make the chemotherapy less than satisfactory. The rapid development of alternative therapies such as DNA vaccines carrying HIV genes, designed zinc finger, and RNA interference remains critically important (Ref. 2 and references therein).

The molecular mechanism of the regulation of HIV-1 transcription provides a molecular basis for developing novel antiviral agents. There is a great need for developing therapeutic agents that can repress transcription of the HIV-1 to overcome the problem viral resistance, because such agents would prevent production of the genetic material for viral replication and the template for reverse transcriptase. Because Sp1 and Tat (*trans*-activator protein) play a central role in the transcription, inhibition of both Sp1 and Tat can repress transcription and replication of HIV-1 (3).

Zinc finger domains are small DNA-binding modules (about 30 a.a.) that can be engineered to bind to the desired target sequences (4–6). Recently, the engineered zinc finger or its fusion form with other functional domains have been constructed by several groups, including our group, and these zinc fingers have successfully activated or repressed gene targets in various systems including the HIV-1 viral gene (Refs. 2, 7, and 8 and references therein). In the case of HIV-1, the engineered zinc finger proteins were designed to bind a key regulatory DNA sequence within the HIV-1 LTR promoter, including Sp1 sites, the TATA box, and the downstream sequences (2, 7, 8). They were even more effective when linked with the repression domain of the SID or KRAB transcription repressors (2, 8). The zinc finger proteins have the potential to exploit several HIV-specific processes, both at the level of transcription at the promoter and also at other nucleic acid-protein interactions such as TAR-Tat interaction. HIV-1 encodes a regulatory protein Tat, which functions by interaction with the specific RNA element called TAR. The protein-RNA interactions are abso-

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; AZF, artificial zinc finger; FBI-1, factor binding to the inducer of short transcript; POZ, pox virus zinc finger; P-TEFb, positive transcription elongation factor b; TAR, *trans*-activator-responsive element; TatdMt, a mutant form of Tat; LTR, long terminal repeat; a.a., amino acid(s); EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase.

lutely required for HIV replication and transcription, and so they are enticing potential target points for antiviral strategies. No studies using zinc finger proteins have yet targeted the Tat-TAR interaction.

The POZ domain of FBI-1 has recently been shown to bind to the zinc finger DNA-binding domain of Sp1 to prevent the recognition of the target GC boxes and thus to repress transcription activation (9). FBI-1 has both zinc fingers recognizing the LTR inducer of short transcript sequences and Sp1-inhibiting POZ domain. In addition, FBI-1 was shown to interact with Tat via its zinc finger and to form an inducer of short transcript-FBI-1-Tat-P-TEFb complex *in vivo*, which suggested that the FBI-1 protein is in close contact with other regulatory proteins and nucleic acids (10, 11).

Initially, we designed transcription repressors that incorporated part or the full length of FBI-1. In addition to the unique features of the FBI-1, we incorporated a novel feature of the TatdMt, the dominant negative mutant form of Tat, for targeting the Tat-TAR interaction. This particular form of the Tat mutant can bind TAR efficiently, but it is lacking the ability to form P-TEFb (12). FBI-1 or POZ-TatdMt fusions can target both the Sp1 and Tat-TAR interaction, and so they are effective transcription blockers. By structural analysis of the fusion gene construct FBI-1-TatdMt, we mapped the functional domains important in transcription repression. Furthermore, we designed artificial zinc finger (AZF) fusions specifically targeting the Sp1-binding GC box region and Tat-TAR interaction. The TatdMt fusion proteins of ZFs were efficiently targeted to the HIV-1 LTR and strongly repressed transcription and replication.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—The HIV-1 LTR-CAT fusion plasmid (pU3R-IIICAT or luciferase) has been described previously (13). The expression plasmids for TatWt (HIVHBX2R type, 86 a.a.), TatdMt (Tat K28A, K50A, 72 a.a.), and FBI-1 were prepared by subcloning their cDNAs into pcDNA3.0 (Invitrogen) (12–14). The mammalian expression plasmids for FBI-1 or POZ domain fusion proteins with a mutant TatdMt were constructed by subcloning their cDNAs into pcDNA3.0-TatdMt plasmid (FBI-1, GenBank™ accession number, AF097916). The pG5-luciferase was from Promega, and the pG5-Sp1-luciferase was prepared by subcloning five copies of the Sp1-binding GC box into the pG5-luciferase. pBind Gal4-POZ_{FBI-1}, and -FBI-1 were prepared by subcloning the cDNA into the pBind plasmid (Promega). All of the constructs were verified by sequencing.

Cell Culture and Transient Transfection Assays—To analyze the repression by Gal4-POZ domain or FBI-1 fusions on Sp1-dependent transcription, CV-1 cells were transfected with a mixture of pG5-Luc or pG5-Sp1-luc plasmid (0.4 μg), pCMV-*LacZ* plasmid (0.2 μg), and various pGal4-POZ_{FBI} or pGal4-FBI (0.4 μg) by using Lipofectamine Plus (Invitrogen). To assay the repression caused by various fusion proteins on HIV-1 transcription, the stable HeLa cells integrated with the HIV-1 LTR-CAT gene (pU3R-III CAT) were transfected with the pcDNA3.0 TatWt expression plasmid (0.3 μg) and various pcDNA3.0 TatdMt fusion expression plasmids (0.3 μg). For the titration experiments, 0–0.3 μg of pcDNA3.0 expression plasmids for FBI-1-TatdMt was cotransfected with pcDNA3.0 TatWt (0.3 μg) into stable HeLa cells. The cells were grown for 24 h and then assayed for reporter gene expression. Variations in transfection efficiencies were normalized using coexpressed β-galactosidase activity.

HIV-1 Replication Inhibition Analysis—Transient *trans*-complementation or a single-round HIV-1 replication inhibition analysis was carried out as described elsewhere (15–18). A plasmid that expresses the HIV-1 genome with the hygromycin B resistance gene was cotransfected into 293T cells with a viral envelope protein VSV-G expression plasmid and either the control (pHYK) or the TatdMt fusion protein expression plasmid. The cotransfection resulted in the production of HIV-1 virus particles that undergo only a single round of infection in susceptible cell lines such as HeLaT4 and Magi cells (15–18). The efficiency of a single round of infection was determined by scoring the numbers of Hyg^R HeLaT4 cells or β-galactosidase-positive Magi cells (see Fig. 2*A*). To produce HIV-1 virus, 293T cells were transfected with HIV-1 proviral DNA (YK177–86, 1 μg) (16), and either the control (pHYK, 2 μg) or TatdMt fusion expression plasmid (fused with POZ domain, FBI-1, artificial zinc finger, 2 μg), the envelope protein expression plasmid (pVSV-G, 0.5 μg), and the *LacZ* gene expression plasmid (MFG/*LacZ*/Puro, 0.5 μg) using Lipofectamine Plus. The MFG/*LacZ*/Puro plasmid was cotransfected to normalize variations in transfection efficiencies. The transfected cells were further incubated for 2 days to prepare a supernatant containing infectious HIV-1 virus particles. The supernatant from the transfected 293T cells was collected, filtered, diluted serially, and used to infect $CD4^+$ HeLaT4 cells or Magi cells (HeLaT4 cells integrated with the HIV-1 LTR-*LacZ* gene). After 4 h, fresh culture medium was then added, and the cells were cultured for selection of Hyg^R-positive cells, and the virus titer was determined by counting the colonies that were fixed and stained with 0.5% crystal violet/50% (v/v) methanol. In the case of Magi cells, the virus titer was determined by scoring the number of Magi cells that turned blue after β-galactosidase staining. The assays results are presented as averages of three independent experiments.

Mapping of Functionally Important Functional Domains of FBI-1-TatdMT Fusion—Various deletion and zinc finger mutants of FBI-1 were prepared by subcloning FBI-I or FBI-1 zinc finger 1 mutant cDNA fragments that had gone through PCR into the pcDNA3.0-TatdMt plasmid (see Fig. 3*A*) (9, 11). For the PCR to amplify the region between a.a. 124 and 584 for the constructs 2-1 and 2-2 (see Fig. 3*A*), 5′-ACGTAAGCTTACCATGGCGCCGACCTCCTGGACCGG-3′ (forward) and 5′-GATCGAATTCGGCGAGTCCGGCTGTGAAGTT-3′ (reverse) were used. To amplify the region between a.a. 124 and 512 for the constructs 3-1 and 3-2, 5′-ACGTAAGCTTACCATGGCGCCGACCTCCTGGACCGG-3′ (forward) and 5′-GATCGAATTCCGGGCTGGGGTCGGGCGCCCCGC-C-3′ (reverse) were used. Also, for the PCR between a.a. 335 and 584 (for 4-1 construct) or a.a. 512 (for 5−1 and 5-2 constructs), 5′-ACGTAAGCTTACCATGGGGGACAGCGACGAGTC-3′ (forward) was paired with reverse primer (5′-GATCGAATTCGGCGAGTCCGGCTGTGAAGTT-3′ or 5′-GATCGAATTCCGGGCTGGGGTCGGGCGCCCCGCC-3′). For the 4–1ZFC, forward primer (5′-ACGTAAGCTTACCATGGGGGACAGCGACGAGTC-3′) was paired with reverse primer (5′-GATCGAATTCGGCGAGTCCGGCTGTGAAGTT-3′) for the PCR. For the PCR of ZF (a.a. 371–495), forward primer (5′-ACGTAAGCTTACCATGGAGAAGGTGGAGAAGATCCGA-3′) and reverse primer (5′-ACGTAAGCTTCGAGGGGACGCCGTTGCAGCC-3′) were used.

PCR was performed by denaturing at 94 °C for 5 min and 30 cycles of an amplification reaction (94 °C 30 s, 60 °C 1 min, 72 °C 3 min), and a final extension was done at 72 °C for 4 min. For PCRs to prepare constructs 1-1, 2-1, 3-1, 4-1, 5-1, 4-1ZFC, and ZF fragments, we used the FBI-1 cDNA that we isolated previously as a PCR template (9). For PCRs of 2-2, 3-2, and 5-2, we used the FBI-1 cDNA with a mutation at the first zinc finger (C384A,C387A) (11). The amplified PCR products were cloned into the pcDNA3.0TatdMt/HindIII-EcoRI plasmid to generate various deletion mutants of FBI-1-TatdMt. ZF cDNA of FBI-1 that had gone through PCR was prepared in the same manner and cloned into pcDNA3.0 HindIII.

Design and Preparation of AZFs Recognizing Various Regions of HIV-1 LTR—Ten AZFs that recognize the HIV-1 LTR were prepared by a method previously described and cloned in pcDNA3.1 (4). The AZFs contain three or four zinc fingers and recognize a 9- or 12-bp target sequence, respectively. ZF-56-II is a four-zinc finger polypeptide, and all others are three zinc fingers. The numbers are the uppermost nucleotides of the recognition sites. These AZFs were designed to recognize the regions around Sp1-binding sites, TATA box, and a downstream region that are critical in the transcription of HIV-1 LTR. All of the AZFs contain HA tag at the N terminus and nuclear localization sequence fused with the zinc finger open reading frame. The AZF expression vectors contain the same 5′ and 3′ sequences flanking the AZF open reading frame.

To prepare the pcDNA3.0-artificial zinc finger fusion TatdMt constructs, 5′-GATCGGTACCATGGAATTGCCTCCAAAAAAGAAG-3′ (forward) and 5′-GATCGATATCTGCGGCCGCTTTTTCACCGGTA-TG-3′ (reverse) were used to subject the zinc finger open reading frame sequences to PCR using various artificial zinc finger plasmids as PCR templates. PCR was performed by denaturing at 94 °C for 5 min, 30 cycles of amplification reaction (94 °C for 30 s, 60 °C for 1 min, and 72 °C for 3 min), and a final extension at 72 °C for 4 min. The PCR products were digested with HindIII and EcoRV and then cloned into the pcDNA3.0-TatdMt plasmid that was digested with HindIII-EcoRV.

Electrophoretic Mobility Shift Assays (EMSA) of AZFs and Specificity of AZF Binding—For EMSA, HA-AZF LTR-62 and HA-AZF LTR-65 polypeptides were prepared using TnT *in vitro* transcription and translation system (Promega, Madison, WI). Antibody against HA polypeptide was purchased from Upstate Biotechnology, Inc. Sp1 was purchased from Promega. Oligonucleotide probes used are (only top strand

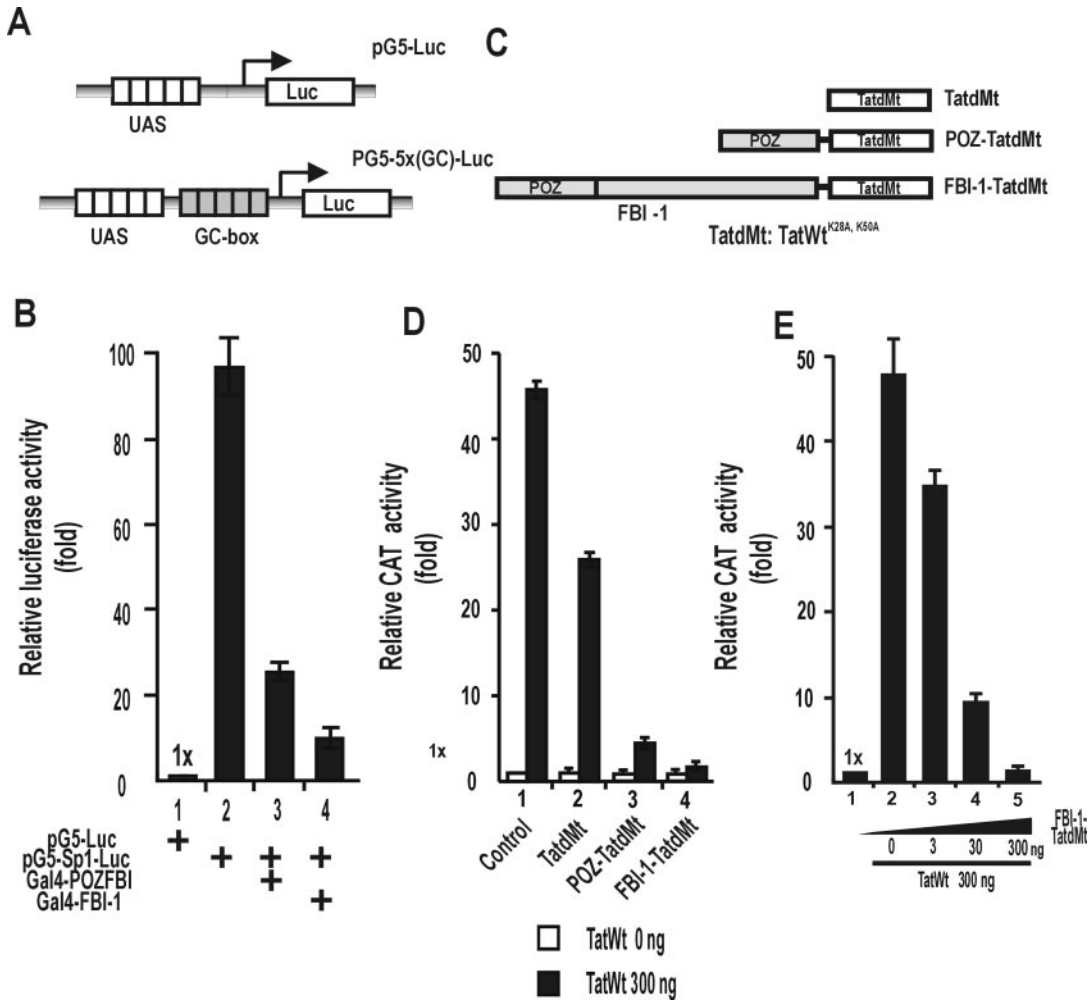


FIG. 1. **Targeting the Sp1-inhibiting polypeptides proximal to Sp1-binding GC boxes potently represses transcription.** *A*, structures of Gal4 UAS or UAS-5x(GC)-luciferase reporter plasmids. *B*, pG5-Luc with no Sp1-binding GC box sites shows minimal promoter activity. Endogenous Sp1 potently activates transcription by more than 95-fold by binding to the GC boxes of pG5–5x(GC)-Luc. The coexpression of Gal4-POZ or FBI-1 fusion potently represses transcription. *UAS*, upstream activator sequences of binding sites for Gal4 fusion proteins. *GC-box*, Sp1-binding site. *C*, structures of TatdMt fusion polypeptides. *D*, transient transfection assays and transcription repression by TatdMt and its fusions in stable HeLa cells integrated with HIV-1 LTR. □, transient transfection in the absence of TatWt. ■, assays in the presence of TatWt. *E*, titration of transcription repression by FBI-1-TatdMt fusion. FBI-1-TatdMt competed with TatWt and potently inhibited transcription activation by TatWt. The average of three independent assays is shown. The *error bars* represent the standard deviations.

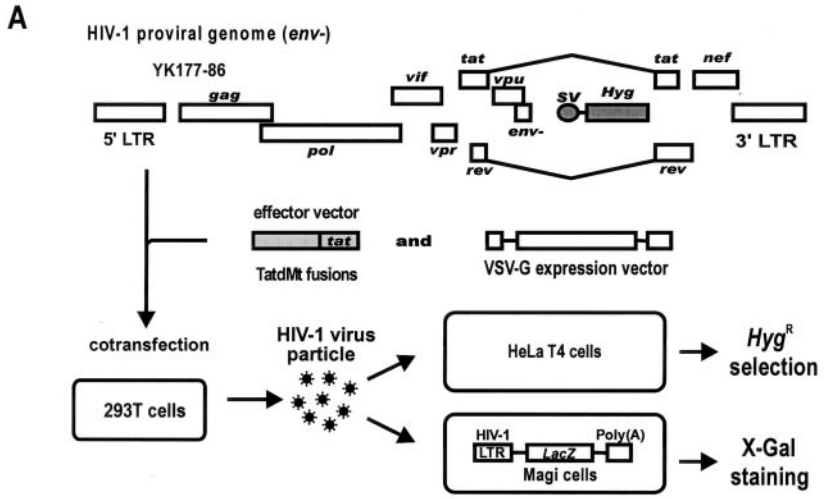
shown): for Sp1 binding, 5′-GATCATTCGATCGGGGCGGGGCGAGC-3′; for HA-AZF LTR-62, 5′-GATCATTCGATCCGGGACTGGCGAGC-3′; and for HA-AZF LTR-65, 5′-GATCATTCGATCGGGCGGGACGCAGC-3′. EMSA was carried out as described previously (9). To show that the AZFs act specifically on their own target sequences and do not act on the Sp1 binding GC box, two well characterized Sp1-dependent human gene promoters fused with reporter luciferase gene, p*ADH5/FDH*-luc and $p21^{Waf/cip1}$-luc, were cotransfected with AZF expression vectors into HeLa cells and analyzed as described above under "Cell Culture and Transient Transfection Assays."

Transcription Inhibition Assays with the Artificial Zinc Fingers or Artificial Zinc Fingers Fused with POZ Domain or TatdMt and Transient Transfection Reporter CAT Assays—To investigate the transcription inhibition by the artificial zinc fingers proteins, the stable HeLa cells integrated with the HIV-1 LTR CAT gene were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 μg/ml) (Invitrogen). After growing the cells for 24 h, the cells were transiently transfected with 0.3 μg of each pcDNA 3.1 zinc finger expression plasmid, 0.3 μg of pcDNA3.0-TatWt expression plasmid, and 0.1 μg of pCMV-β-galactosidase (*LacZ*) mixed with Lipofectamine Plus (Invitrogen), and the cells were then allowed to grow for 24 h. The rest of the assay procedures are the same as described above in the transient transfection reporter assays.

To investigate whether the transcription activation by Sp1 and CyclinT1 can be inhibited by the artificial zinc fingers and the AZFs fused with POZ domain and TatdMt, the stable HeLa cells were transfected with a plasmid mixture composed of 0.3 μg of pcDNA3.0 AZF LTR-65 or AZF LTR-65-TatdMt, in the presence or absence of 0.3 μg of pCMV-Sp1 and/or cyclin T1, and 0.1 μg pCMV-β-galactosidase (*LacZ*) using Lipofectamine Plus. The rest of the transcription assay procedures are the same as described above.

RESULTS

TatdMt fusion protein can potently represses transcription of HIV-1—FBI-1 has been shown to bind to the zinc finger DNA-binding domain of Sp1, to prevent Sp1 from recognizing the target GC boxes, and to repress transcription activation by Sp1 (9). We tested whether Gal4DBD fusion proteins with FBI-1 or the POZ domain of FBI-1 could repress transcription activation by Sp1 when these proteins were targeted proximal to the GC boxes. Targeting Sp1-inhibiting polypeptides using Gal4-DBD proximal to the Sp1 binding GC box can effectively repress transcription by more than 75% (Fig. 1, *A* and *B*). We applied this finding for the control of HIV-1 transcription. Instead of Gal4DBD, we used TatdMt (72 a.a. Tat K28A,K50A), a mutant form of TAR RNA-interacting polypeptide Tat that lacks the ability to interact with P-TEFb but binds efficiently to TAR (12). The two lysine residues at a.a. 28 and 50 that are critical in the interaction with cyclin T1 were replaced with alanines in TatdMt. TatdMt neither forms a complex with P-TEFb (cyclin



B

Effector plasmid	Number of Hyg^R colonies ± SD /ml	Fold decrease
pHYK (negative control)	$1.6 \pm 1.5 \times 10^5$ cfu /mL	–
FBI-TatMt	$5.6 \pm 0.6 \times 10^2$ cfu /mL	286

FIG. 2. **FBI-TatdMt fusion protein potently represses HIV-1 replication.** *A*, schematic diagram of HIV-1 replication inhibition assay. HIV-1 proviral DNA, YK177–86, was cotransfected with an envelope protein VSV-G expression plasmid and the control pHYK plasmid or the effector TatdMt fusion plasmids into 293T cells. Cell-free medium was collected, diluted, and used to infect HeLaT4 cells. The number of infected cells was scored as described under "Experimental Procedures." *B*, FBI-1-TatdMt reduced the number of infectious HIV-1 virus particle by 286-fold compared with negative control PHYK. The results are the averages of three independent experiments.

T1 and cyclin-dependent protein kinase 9) nor activates transcription. These features of TatdMt can be useful in targeting Sp1-inhibiting polypeptides to HIV-1 LTR. TAR-bound Tat is in close proximity to and interacts with cellular proteins such as Sp1, NF-κB, general transcription factors (TFIID and TFIIH), and P-TEFb (1, 3, 14, 19).

We fused the Sp1-inhibiting POZ domain and FBI-1 with either the N or C terminus of TatdMt (72 a.a.) (Fig. 1*C* shows the constructs in one configuration). The fusion expression plasmids were transfected into stable HeLa cells with the integrated HIV-1 LTR-CAT gene in the presence or absence of TatWt expression. In the absence of TatWt, transcription in the stable HeLa cells occurred at a low basal level (Fig. 1*D*, *lane 1*). TatWt potently activated the transcription of HIV-1 LTR by 46-fold (Fig. 1*D*, *lane 1*, *filled bar*). In contrast, TatdMt or TatdMt fusions alone did not activate transcription over the basal level (Fig. 1*D*, *open bars*, compare *lanes 1* and *lanes 2–4*). However, TatdMt reduced transcription activation by TatWt to half of that observed with TatWt alone, and this suggested that TatdMt competes with TatWt for TAR (Fig. 1*D*, compare *lanes 1* and *2*, *filled bars*). The TatdMt fusions successfully competed with TatWt for TAR and potently repressed transcription (Fig. 1*D*, compare *filled bars*, *lanes 1 versus lanes 3* and *4*). FBI-1-TatdMt was the most potent repressor, and it repressed transcription down to the basal transcription level even in the presence of an excess of TatWt expression plasmid (0.3 μg) (Fig. 1*D*, *filled bar*, *lane 4*). The POZ domain (120 a.a.) is mainly responsible for the repressor activity of the FBI-1-TatdMt fusion. A titration experiment shows that FBI-1-TatdMt competed with TatWt and potently inhibited transcription by 30% at 3 ng and 80% at 30 ng, and it nearly nullified transcription at 300 ng (Fig. 1*F*).

FBI-TatdMt Fusion Protein Potently Represses HIV-1 Replication—We investigated whether TatdMt fusion proteins can inhibit HIV-1 replication by using transient trans-complementation assays in 293T cells (Fig. 2*A*) (15–18). The FBI-1-TatdMt in the configuration, as shown in Fig. 3*A*, reduced the number of infectious HIV-1 virus particles by 286-fold (Fig. 2*B*).

Mapping of Functional Domains of FBI-1-TatdMT Fusion—Encouraged by the above finding, we investigated which domains of FBI-1-TatMt fusion contribute to this potent transcription repression. We prepared various deletion and mutation constructs (Fig. 3*A*). Deletions were made within the POZ domain (1–123 a.a.), C terminus (513–584 a.a.), and junctional domain between the POZ domain and the zinc fingers (124–334 a.a.). In addition, to investigate the importance of the zinc finger DNA-binding domain, mutations at the two cysteine residues of the first zinc finger (C384A and C387A) were introduced (11). Transient transfection assays in the stable HeLa cells with integrated HIV-1 LTR-CAT showed that the FBI-TatdMt again potently repressed transcription activation by TatWt (Fig. 3*B*, compare *lanes 2* and *3*). Deletion of the POZ domain (construct 2-1) resulted in 5-fold decrease in transcription repression, suggesting the importance of the domain in repression by inhibiting Sp1 (Fig. 3*B*, compare *lanes 3* and *4*).

Although deletion of the POZ domain decreased the repression potential of the fusion proteins, the truncated fusions containing zinc fingers and TatdMt still retained a significant repression potential. All of the zinc finger mutants (constructs 2-2, 3-2, and 5-2) showed no repressor activity comparable with any of the deletion mutants. They showed repression activity that was only comparable with TatdMt alone (Figs. 3*B* and 1*E*). This data suggested that the zinc finger is essential in transcription repression of the fusion constructs, and this probably happens by blocking transcription initiation or progression and by helping TatdMt in TAR binding. Our data indicated that the POZ domain and the zinc finger are the important repression domains of FBI-1-TatdMt. The zinc finger itself, with no nuclear localization sequence attached, was able to repress transcription, although relatively weakly compared with either

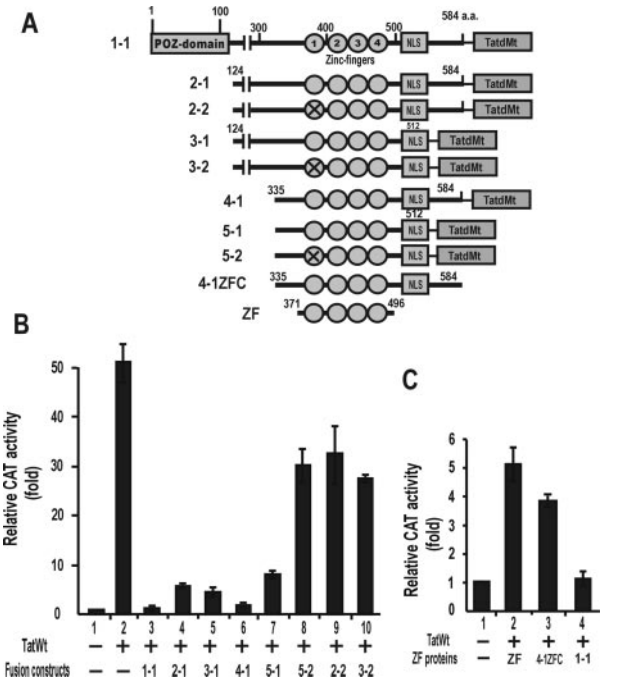
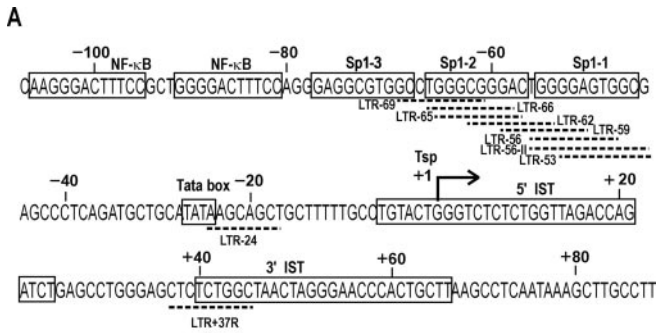
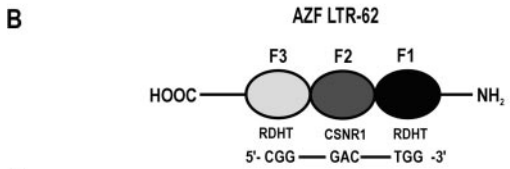


FIG. 3. **Mapping of functional domains of FBI-1-TatdMT fusion important in transcription repression.** *A*, structures of various deletion or mutant constructs of FBI-1-TatdMt fusion protein (construct 1-1). Deletion was made with the POZ domain, C terminus, nuclear localization sequence (*NLS*), and junction domain between the POZ domain and the zinc fingers. Mutations (marked with ×) are at the first zinc finger. *B*, transient transfection assays in the stable HeLa cells. Deletion or mutation of POZ domain, TatdMt, and zinc finger resulted in a significant decrease in transcription repression. *C*, comparison of the constructs showing low promoter activity, zinc finger itself with no nuclear localization sequence, zinc finger plus C terminus (4-1ZFC), and 1-1.





C

	Name	Finger1	Finger2	Finger3	Finger4	Binding sequence
1	LTR-69	RDER2	RDHT	DSCR		5'-GCC TGG GCG-3'
2	LTR-66	QSHR2	REDR2	RDHT		5'-TGG GCG GGA-3'
3	LTR-65	CSNR1	RDHT	RSHR		5'-GGG CGG GAC-3'
4	LTR-62	RDHT	CSNR1	RDHT		5'-CGG GAC TGG-3'
5	LTR-59	QSHR2	RDHT	CSNR1		5'-GAC TGG GGA- 3'
6	LTR-56	REDR2	QSHR2	RDHT		5'-TGG GGA GTG-3'
7	LTR-56II	REDR2	QSHR2	RDHT	DSCR	5'-TGG GGA GTG GCG-3'
8	LTR-53	REDR2	RDER2	QSHR2		5'-GGA GTG GCG-3'
9	LTR-24	VSTR	QSSR1	QSNK		5'-TAA GCA GCT-3'
10	LTR-37R	RSNR	QSHV	DSCR		5'-GCC AGA GAG-3'

FIG. 4. **Design of AZFs recognizing various regions of HIV-1 LTR and transcription repression.** *A*, locations of ten artificial three or four zinc finger-binding sites are indicated with *dotted lines*. The AZF LTR-56 (II) is the only four zinc finger polypeptide. The zinc fingers recognize a 9- or 12-bp nucleotide sequence. The *numbers* of the AZFs are the uppermost nucleotide of the recognition site. The *arrow* (→) and *Tsp*, transcription start point +1. *B*, structure of the representative zinc finger polypeptide composed of three artificial zinc fingers of the AZF LTR-62. Shown are the fingers (*F1*, *F2*, and *F3*), the amino acids of each finger critical in specific target recognition, and the nucleotide sequence that each zinc finger recognizes. *C*, list of artificial zinc fingers, structure of fingers, and nucleotide sequences they recognize.

4-1ZFC or FBI-1-TatdMt. FBI-1-TatdMt repressed transcription five times more strongly as compared with the zinc finger alone. Our data also suggest that although the zinc finger of FBI-1 can repress transcription, the degree of transcription repression can be significantly enhanced by a fusion with the POZ domain and TatdMt (Fig. 3*C*).

Design of AZFs Recognizing Various Regions of HIV-1 LTR and Transcription Repression—Because FBI-1 is ubiquitously expressed and overexpression of the fusion protein in the targeted cells can potentially alter the expression of various genes, we designed and prepared ten artificial zinc fingers that specifically recognized the HIV-1 LTR (Fig. 4, *A* and *C*). The zinc fingers were designed to contain three or four zinc fingers and to recognize 9 or 12 bp, respectively (Fig. 4*C*). Eight of the ten zinc fingers were targeting the Sp1-binding region (−77 to −46 bp), and two others targeted the TATA box or downstream region (+37 to +66 bp) of the transcription start point (Fig. 4*A*).

The zinc finger expression plasmids were cotransfected with the TatWt expression plasmid into stable HeLa cells. Among the AZFs we tested, AZF LTR-62 (recognizes CGGGACTGG) and AZF LTR-65 (recognizes GGGCGGGAC) were stronger transcription repressors than the others and repressed transcription by 88–67%. AZF LTR-62 and -65 were selected for further study (Fig. 5).

AZF LTR-62 and AZF LTR-65 Do Not Bind to the Putative GC Box Recognized by Sp1 and Do Not Affect the Sp1-dependent Transcription of the ADH5/FDH and p21$^{Waf/Cip1}$ Genes—We investigated the specificity of AZF binding by EMSA and transient transfection assays. Sp1 binds to the putative GC box probe, and the Sp1-probe complex is supershifted by rabbit polyclonal antibody against Sp1 (Fig. 6*A*, *lanes 2* and *3*). However, neither *in vitro* translated HA-tagged AZF LTR-62 nor AZF LTR-65 bind to the GC box probe (Fig. 6*A*, *lanes 5* and *7*). AZF LTR-62 and AZF LTR-65 bind their target sequence probes, and the complexes are supershifted by antibody against HA tag (Fig. 6*B*). Once the AZF expression plasmid and the human gene promoter are cotransfected into HeLa cells, the AZFs do not affect the transcription of the two well characterized Sp1-dependent human promoters, *ADH5/FDH* and p21$^{Waf/Cip1}$, suggesting that the AZFs act specifically only on their own recognition sites (Fig. 6, *C* and *D*).

AZFs Targeted to Sp1-binding Region Can Repress the Transcription Activation by Cyclin T1 and Sp1—It is interesting that the two strong transcription repressors AZF LTR-62 and AZF LTR-65 recognize the region around the Sp1-binding GC box, one of the key regulatory elements of HIV-1 transcription (19). Sp1 can activate transcription by interacting with cofactors, the components of the general transcription machinery, and cyclin T1 (20, 21). In particular, Sp1 binding and the

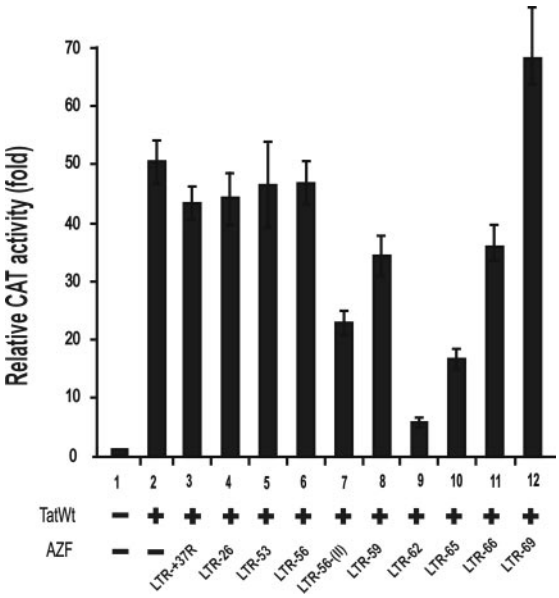


FIG. 5. **Transcription repression by the AZFs recognizing various regions of HIV-1 LTR.** The ten zinc fingers were designed to bind to the Sp1-binding region (−77 to −46 bp), the TATA box, and the downstream region (+37 to +66 bp) of transcription start point. The zinc finger expression plasmids were cotransfected with the TatWt expression plasmid into stable HeLa cells integrated with HIV-1 LTR fused with CAT reporter gene. Among the AZFs tested, AZF LTR-62 and AZF LTR-65 showed relatively stronger transcription repression than the others.

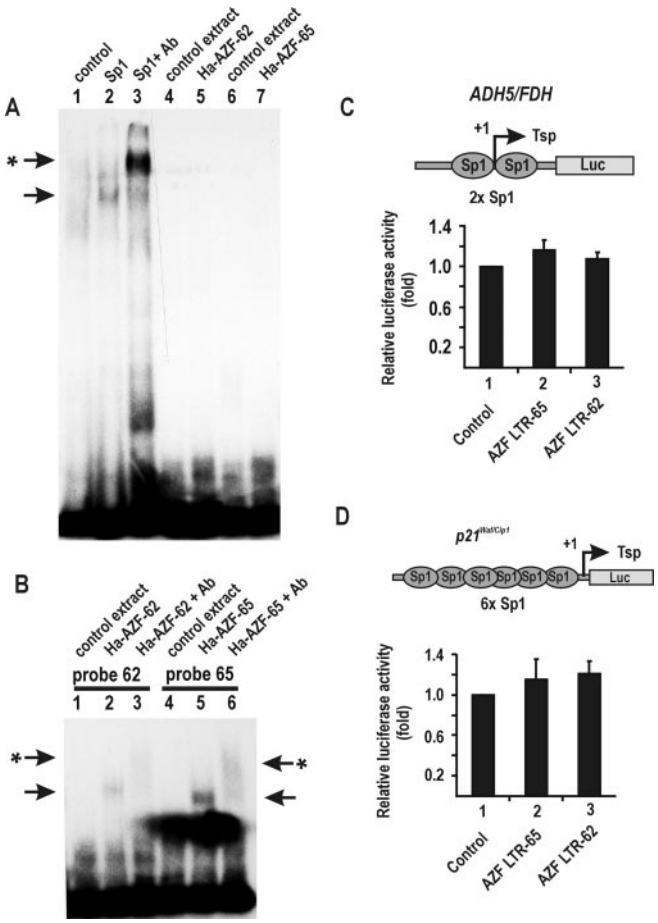


FIG. 6. **The AZFs do not bind the Sp1-binding GC box and do not affect transcription of Sp1 dependent human gene promoters, *ADH5/FDH* and p21$^{Waf/Cip1}$.** *A*, EMSA with [α-^{32}P]dCTP labeled GC box probe. Sp1 binds to the probe, which is supershifted by antibody against Sp1. Neither *in vitro* translated AZF LTR-62 nor AZF LTR-65 bind to the GC box probe. *Control*, control *in vitro* translated reticulocyte extract. *B*, both *in vitro* translated HA-tagged AZF LTR-62 and AZF LTR-65 bind to their own target [α-^{32}P]dCTP labeled oligonucleotide probes and supershifted by HA antibody. *C* and *D*, neither AZF LTR-62 nor AZF LTR-65 affect the transcription of the two well characterized Sp1-dependent human gene promoters, *ADH5/FDH* and p21$^{Waf/Cip1}$. The *arrows* indicate retarded [α-^{32}P]dCTP-labeled oligonucleotide probed bound by Sp1 or synthetic AZFs. The *asterisk* indicates the supershifted protein-probe complex by antibody. *Luc*, luciferase. The *arrow* and *Tsp* indicate the transcription start point +1.

interaction between Sp1-cyclin T1 are important for transcription of HIV-1 LTR in the absence of Tat (Fig. 7*A*). We tested whether the AZF or AZF-TatdMt fusion can repress transcription contributed by the Sp1-cyclin T1 interaction. In stable HeLa cells transfected with various expression vectors for cyclin T1, Sp1, AZF LTR-62, or POZ-AZF LTR-62-TatdMt fusion proteins, cyclin T1 activated transcription by 5.5-fold, suggesting that the molecular interaction does exist and is important in the transcription activation in the absence of TatWt. The transcription activation by Sp1 and cyclin T1 can be effectively repressed by AZF LTR-62 or POZ-AZF LTR-62-TatdMt (Fig. 7*B*, *lanes 2–4*). Ectopic Sp1 slightly activates transcription over the basal level, and cyclin T1 further activates it by 5-fold. Transcription activation by cyclin T1 can be blocked by the AZF LTR-62 alone and POZ-AZF LTR-62-TatdMt fusion (Fig. 7*B*, *lanes 6–8*). The data suggests that the AZF LTR-62 bound at the region around the Sp1-binding GC box inhibits Sp1-cyclin T1-dependent transcription.

POZ domain, TatdMt Fusion Proteins of AZF LTR-62 and AZF LTR-65 Potently Repress Transcription and HIV-1 Replication—We found that the functional domains important in transcriptional repression by FBI-1-TatdMt are the POZ domain, zinc fingers, and TatdMt (Fig. 3). Fig. 5 showed that although the AZF can repress transcription, it alone could not repress transcription quite down to the basal level. Therefore, we fused the POZ domain and TatdMt with AZF LTR-62 and AZF LTR-65 and then tested their activities in transcription repression (Fig. 8). The AZF alone can repress transcription down to about 20% of the transcription level activated by TatWt. By being fused with TatdMt, the fusion AZF became a 2–3-fold more potent transcription repressor. AZF LTR-62-TatdMt or AZF LTR-65-TatdMt became even more potent repressors after both were fused with the POZ domain, and they completely nullified transcription activation by TatWt (Fig. 8*B*, *lanes 6* and *9*).

We also performed *trans*-complementation HIV-1 viral replication inhibition assays using Magi cells as is described under "Experimental Procedures" and Fig. 2*A*. The single round transfection competent HIV-1 viruses were produced by the cotransfection of recombinant HIV-1 genome defective in envelope protein gene, VSV-G envelope protein gene and effector POZ-AZF LTR-62 or -65-TatdMt expression plasmids or control vector plasmid (pYHK) into the virus packaging 293T cells. The viral supernatant was diluted and then used to infect the HeLaT4 cells and to count the number of HeLaT4 cells infected with HIV-1 virus; the cells were maintained in hygromycin-containing medium. Although FBI-1-TatdMt potently inhibited HIV-1 replication, both the POZ domain-AZF LTR-62-TatdMt and POZ domain-AZF LTR-65-TatdMt inhibited replication much more potently, by as much as 4,675-fold (Table I). Our data suggest that the newly designed AZF LTR-TatdMt fusion proteins are much more effective in replication inhibition compared with FBI-1-TatdMt. Our data imply that once the fusion expression plasmids are delivered to HIV-1-infected cells and expressed effectively, the fusion protein can potently inhibit HIV-1 virus transcription and replication.

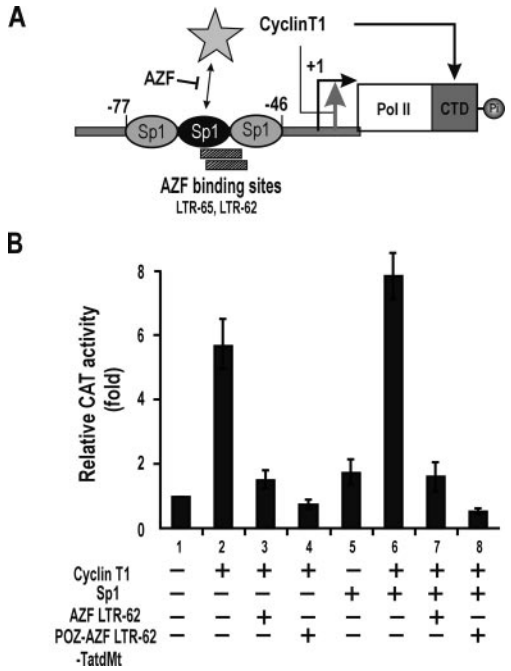


FIG. 7. **The AZF LTR-62 targeted adjacent to the middle Sp1-binding site can repress the transcription activation contributed by cyclin T1 and Sp1 interaction.** *A*, diagram on the role of cyclin T1(⋆), Sp1, and RNA polymerase II in the transcription activation of HIV-1. Promoter-bound Sp1 recruits cyclin T1. Cyclin T1 and interacting kinase phosphorylate the C-terminal domain (*CTD*) of RNA polymerase II and activate transcription. *B*, transient transfection assays in stable HeLa cells. Transcription activation by Sp1 and cyclin T1 can be blocked by AZF or AZF fusion. *Tsp*, transcription start point; *Pol II*, RNA polymerase II.

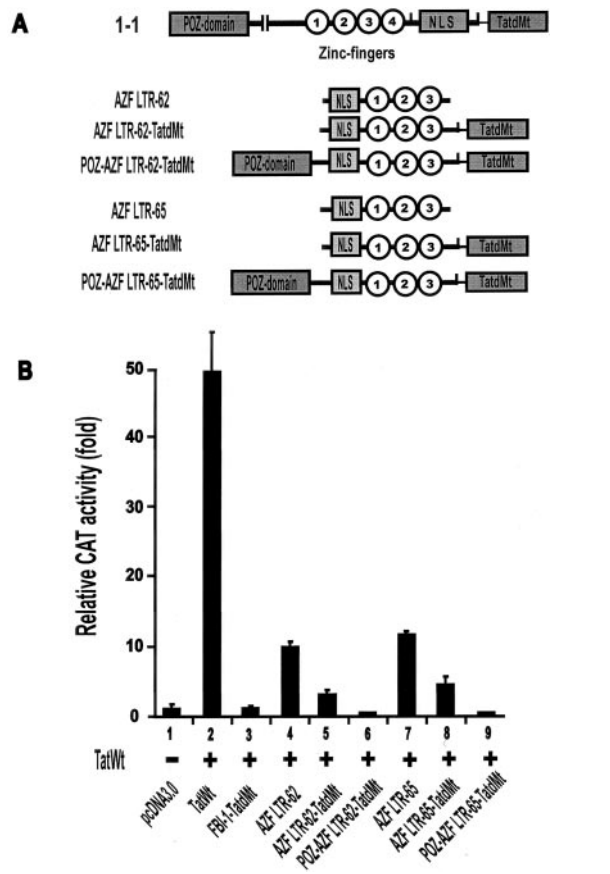


FIG. 8. **POZ domain, TatdMt fusion proteins of artificial zinc fingers (AZF LTR-62 and AZF LTR-65) recognizing the Sp1-binding sites (−62 to −54 bp and −65 to −57 bp) potently repress transcription.** *A*, structures of artificial zinc fingers, AZF-LTR-62, AZF-LTR-65, and their fusions as compared with FBI-1-TatdMt (construct 1-1). *B*, transient transfection assays in stable HeLa cells. Transcription activation by TaWt can be repressed by AZF or AZF fusions. POZ-AZF-LTR-62 or -LTR-65-TATdMt repressed transcription below basal transcription level. *NLS*, nuclear localization sequence.

DISCUSSION

The molecular mechanism of the regulation of HIV-1 transcription provides a molecular basis for developing novel antiviral agents. Based on the molecular mechanism of transcription regulation on HIV-1 LTR, we developed artificial zinc fingers targeted to the high affinity Sp1-binding site, and by being fused with TatdMt and POZ domain, the zinc fingers strongly block both Sp1-cyclin T1-dependent transcription and Tat-dependent transcription, even in the presence of excess expressed Tat.

Of the several key target sites on the HIV-1 LTR for designed zinc fingers including ours, the most effective ones are the one targeted to the Sp1-binding region located at −46 and −77 (relative to the RNA start site, +1) (2, 7, 8). The three Sp1-binding sites are particularly importance in transcription (19). The artificial zinc fingers developed by other researchers targeted to the Sp1 sites are also very effective in transcription repression, particularly by being fused with the repressor domain of KRAB or SID (2, 7, 8). In our case too, among the ten zinc fingers designed, AZF LTR-62 and AZF LTR-65 targeted to the GC box region were particularly effective transcription repressors, both for the zinc finger alone and particularly in the fusion form with POZ domain and TatdMt. Although the direct comparison of the zinc finger in transcription repression is difficult because investigators are using different assay systems and cell lines, the zinc fingers developed by us are also quite potent repressors, and they nullify transcription and viral replication as much as 4,675-fold. The binding sequences of AZF LTR-62, and AZF LTR-65 are partially overlapping with the Sp1-binding site but do not match precisely with Sp1-binding sites of HIV-1 LTR. The AZFs do not recognize the GC box recognized by Sp1 and do not alter expression of the genes controlled by Sp1 such as *ADH5*/*FDH* and $p21^{Waf/Cip1}$.

TABLE I
Inhibition of HIV-1 replication by TatdMt fusion proteins of artificial zinc fingers

Expression plasmids encoding TatdMt fusion proteins of artificial zinc fingers were cotransfected with a HIV-1 proviral DNA (YK177–86) and a VSV-G expression plasmid, and virus-containing medium was assayed for virus titration as described under "Experimental Procedures."

Effector plasmid	Number of *Hyg*^{r} colonies ± S.D./ml	Fold decrease
	cfu/ml	
pHYK (negative control)	$3.6 \pm 0.7 \times 10^5$	
FBI-TatMt	$1.3 \pm 1.1 \times 10^3$	277
POZ-AZF LTR-62-TatMt	$5.7 \pm 1.2 \times 10^2$	632
POZ-AZF LTR-65-TatMt	$7.7 \pm 1.5 \times 10^1$	4,675

Sp1 bound at the proximal promoter of HIV-1 was shown to interact with cyclin T1, and the interaction was important in basal transcriptional activation of HIV-1 in the absence of Tat (20, 21). We investigated whether AZF LTR-62 or -65 alone or in fusion forms with the TatdMt could inhibit Sp1-cyclin T1-dependent activation and found that the designed ZFs repressed the activation contributed by the interaction. The data may explain why the several zinc fingers targeted to the Sp1-binding regions are quite efficient transcription repressors (2, 8). Although targeting the zinc finger adjacent to the middle Sp1-binding site can effectively blocked transcription by Sp1 and cyclin T1, it may also be important to suppress the Sp1

activity sitting slightly upstream and downstream of the middle Sp1 site (−66 to −57 bp). We have previously shown that the POZ domain targeted proximal to the Sp1-binding site can prevent the Sp1 binding because the POZ domain can interact with incoming Sp1 and prevent the target recognition (9). Also, POZ domain can interact with histone deacetylase and corepressors (mSin3A, NCoR/SMRT, and BCoR), and this interaction can make nearby nucleosomes compact by recruited histone deacetylase (22, 23). The nucleosome compaction around the middle Sp1-binding site may prevent Sp1 and NF-κB from binding to the regulatory sequences. This may explain why the AZF LTR-62 and LTR-65 are much more effective transcription repressors by being fused with POZ domain and TatdMt.

No studies using AZF fusion have yet targeted the Tat-TAR interaction, so we fused the above POZ-AZF fusions with the polypeptide targeting this unique interaction, using TatdMt that completely lacked interaction with cyclin-dependent protein kinase 9-cyclin T1, but it retained a good TAR binding activity (12). The fusions were found to be potent repressors both in transcription and replication probably by successful blocking of transcription activation by both Sp1-cyclin T1 and Tat-TAR-cyclin T1 interactions. Our AZF fusions are different from other zinc fingers in that they contain Sp1 inhibiting the POZ domain, a designer zinc finger, and a TatdMt. The AZF fusions have been targeted specifically, and they completely blocked both transcription of integrated HIV-1 viral gene and replication. The fusions almost completely blocked the transcription by Sp1, Tat, and cyclin T1. Accordingly, we suspect that the transcription activation by NF-κB will be also blocked because basal transcription mainly contributed by Sp1 is essentially blocked at the transcription initiation stage, as was previously suggested by other zinc finger proteins targeting the GC boxes (2, 7).

The zinc fingers developed by others and ourselves were targeted to the key regulatory elements of HIV-1 gene expression. The key regulatory elements are critical for viral transcription and replication and less likely to have mutation in the region. For example, base substitution mutations targeted to one or all three Sp1-binding sites were found both to eliminate the binding of Sp1 and to cause up to a 10-fold reduction in transcriptional efficiency *in vitro* (19). Moreover, HIV-1 viruses with mutation in the critical regions such as the high affinity Sp1-binding GC box and TAR are not likely to be replication-competent. If there is a HIV-1 subtype with mutation in the region we are targeting, it will be still possible to design a new artificial zinc finger and repress transcription effectively by incorporating the POZ domain and TatdMt. However, the development of resistance to zinc finger transcription factors can also be anticipated, and an effective therapeutic strategy based on ZF may require the delivery of multiple transcription factors.

Highly active antiretroviral therapy is a highly effective in the treatment of HIV-1 disease. However, virus resistance, toxicity, side effects, and patient compliance are problematic for many HIV-1 patients (Ref. 24 and references therein). Although the effects of highly active antiretroviral therapy are often immediate and profound, the virus is not eliminated completely, and latently infected cells do persist (25, 26). Virus replication rebounds rapidly after drug withdrawal. The other therapeutic strategies such as inhibition of viral transcription such as antisense oligonucleotides, small interference RNA (27–29), and ribozymes (30, 31) have long been recognized as an important goal in HIV-1 therapy. In this study, based on the molecular mechanism of interaction among regulatory proteins, DNA, and RNA, we developed novel zinc finger fusion proteins that can inhibit transcription. The designed zinc fingers approach targeting the transcription mechanism involving Sp1, GC boxes, cyclin T1, and Tat-TAR interaction may represent a highly effective alternative therapy for HIV infection, along with the development of novel means of specific gene delivery to the HIV-1-infected cells. Our work extends recent studies by Reynolds *et al*. (2) and Segal *et al*. (8), and this work is unique in that we take full advantage of the Sp1-inhibiting POZ domain and the dominant negative mutant form of Tat.

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