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-inhibiting 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-

1 The abbreviations used are: HIV-1, human immunodeficiency virus,
Zinc Finger Fusion Protein Repression of HIV Transcription

lately 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.

Variations in transfection efficiencies were normalized using coexpression vectors containing POZ domain. In addition, FBI-1 was shown to interact with Sp1 recognizing the regions around Sp1-binding sites, TATA box, and a down-stream region that are critical in the transcription of HIV-1 LTR. All of the constructs were verified by sequencing.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Antibodies**—The HIV-1 LTR-CAT fusion plasmid (pU3R-IICAT 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, AF022539; the pG5-luciferase (C-terminal) fusion plasmid was from Promega). FBI-1, artificial zinc finger (AZF) fusion protein was prepared using TNT system (Promega, Madison, WI). Antibody against HA polypeptide was prepared using TNT system (Promega, Madison, WI).

**Cell Culture and Transient Transfection Assays**—To assay the repression of POZ domain on Sp1-responsive transcription, CV-1 cells were transfected with a mixture of pG5-Luc or pG5-Sp1-luc plasmid (0.4 µg), pcDNA3.0 expression plasmids for FBI-1-TatdMt was cotransfected with pcDNA3.0 expression plasmid for FBI-1 or TatdMt fusion protein (Promega). All of the constructs were verified by sequencing.

**HIV-1 Replication Inhibition Assays**—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 Hyg² HeLaT4 cells or β-galactosidase-positive Magi cells (see Fig. 2A). 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 (pSV5-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 Hyg² or TatdMt expression vectors containing POZ domain. In addition, 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.

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-ZFC, and ZF fragments, we used the FBI-1 DNA 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 PCR primer sites, and the FBI-1 cDNA from the other constructs were cloned into the pcDNA3.0 TatdMt/HindIII-EcoRV 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 with ZF-56-II or ZF-56-I, the PCR-amplified fragments 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

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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, pADH5/FDH-luc and p21Waf/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 pcDNA3.1 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 average of three independent assays is shown. The error bars represent the standard deviations.

**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 when these proteins were targeted proximal to the GC boxes. Targeting Sp1-inhibiting polypeptides using Gal4-DBD proximal to the GC boxes of pG5–5x(GC)-Luc 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
Zinc Finger Fusion Protein Repression of HIV Transcription

**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 particles 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-kB, general transcription factors (TFIID and TFIH), 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. 1C shows the constructs in one configuration). The fusion expression plasmids were transfected into stable HeLa cells with 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. 1D, lane 1). TatWt potently activated the transcription of HIV-1 LTR by 46-fold (Fig. 1D, lane 1, filled bar). In contrast, TatdMt or TatdMt fusions alone did not activate transcription over the basal level (Fig. 1D, 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. 1D, compare lanes 1 and 2, filled bars). The TatdMt fusions successfully competed with TatWt for TAR and potently repressed transcription (Fig. 1D, 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. 1D, 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. 1F).

**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. 2A) (15–18). The FBI-1-TatdMt in the configuration, as shown in Fig. 3A, reduced the number of infectious HIV-1 virus particles by 286-fold (Fig. 2B).

**Mapping of Functional Domains of FBI-1-TatdMt Fusion**—Encouraged by the above finding, we investigated which domains of FBI-1-TatdMt fusion contribute to this potent transcription repression. We prepared various deletion and mutation constructs (Fig. 3A). 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. 3B, 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. 3B, 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. 3B and 1E). 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
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/GC Box—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
interaction between Sp1-cyclin T1 are important for transcription of HIV-1 LTR in the absence of Tat (Fig. 7A). 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. 7B, 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. 7B, 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. 8B, 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. 2A. 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.

**References**

1. Experimental Procedures and Fig. 2A. 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.
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 adjacent to the middle Sp1-binding site located at −62 to −54 bp and −65 to −57 bp (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 Tat-dependent transcription, even in the presence of excess expressed Tat.

Expression plasmids encoding 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 Tat can be repressed by AZF or AZF fusion. POZ-AZF-LTR-62 or -LTR-65-TatdMt repressed transcription below basal transcription level. NLS, nuclear localization sequence.

TABLE I

| Effector plasmid   | Number of Hyg' colonies ± S.D/ml | Fold decrease |
|--------------------|----------------------------------|---------------|
| pHyK (negative control) | $3.6 \pm 0.7 \times 10^5$ | 1          |
| 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 binding sites in the proximal promoter of HIV-1.
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 deactylase and corepressors (mSin3A, NCoR/SMRT, and BCoR), and this interaction can make nearby nucleosomes compact by recruited histone deactylase (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 TadMt.

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 TadMt 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 TadMt. 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 TadMt. 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 efforts 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.

REFERENCES

1. Jeang, K.-T., Xiao, H., and Rich, E. A. (1999) J. Biol. Chem. 274, 28837–28840
2. Reynolds, L., Ullman, C., Moore, M., Isalan, M., West, M. J., Clapham, P., and Choo, Y. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1615–1620
3. Frankel, A. D., and Young, J. A. (1998) Annu. Rev. Biochem. 67, 1–25
4. Bae, K. H., Kwon, Y. D., Shin, H. C., Hwang, M. S., Ryu, E. H., Park, K. S., Yang, Y. H., Lee D. K., Lee, Y., Park, J., Kwon, H. S., Kim, H. W., Ye, B. I., Lee, H. W., Sohn, S. H., Yoon, J., Seol, W., and Kim, J. S. (2003) Nat. Biotechnol. 21, 275–280
5. Segal, D. J., and Barbas, C. F. (2003) Curr. Opin. Chem. Biol. 4, 34–39
6. Lee, D. K., Seol, W., and Kim, J. S. (2003) Curr. Top. Med. Chem. 3, 645–657
7. Segal, D. J., Kobayashi, S., and Chen, Y. (2001) Nat. Biotechnol. 19, 656–660
8. Segal, D. J., Goncalves, J., Eberhardy, S., Swan, C. H., Torbett, B. E., and Barbas, C. F. (2004) J. Biol. Chem. 279, 14509–14519
9. Jeang, K.-T., Suh, D., Edenberg, H. J., and Hur, M. W. (2002) J. Biol. Chem. 277, 26761–26768
10. Pendergrast, P. S., Wang, C., Hernandez, N., and Huang, S. (2002) Mol. Biol. Cell 13, 915–929
11. Lescan, M. J., Pendergrast, P. S., Stavropoulos, P., Colmenares, S. U., Kobayashi, R., and Hernandez, N. (1999) Nucleic Acids Res. 27, 1251–1262
12. Rispens, T., Vanhulle, C., Schiltz, L., Adam, E., Xiao, H., Maudoux, F., Calonne, C., Burny, A., Nakatani, Y., Jeang, K.-T., Benkirane, M., and Van Lint, C. (1999) EMBO J. 18, 6106–6118
13. Kodroski, J., Patarca, R., Rosen, C., Wong-Staal, F., and Helsetine, W. A. (1985) Science 299, 74–77
14. Karn, J. (1999) J. Mol. Biol. 29, 235–254
15. Maddon, P. J., Dalglish, A. G., McDougal, J. S., Clapham, P. R., Weiss, R. A., and Axel, R. (1986) Cell 47, 333–348
16. Kim, Y.-S., and Panginan, A. T. (1996) J. Neurovirol. 34, 652–663
17. Engleman, E. G., Ulrich, C. K., Schonenberger, J., Guan, R. K., and Grossman, J. P. (2001) J. Immunol. 167, 2766–2771
18. Kimpton, J., and Emmerman, M. (1992) J. Virol. 66, 2232–2239
19. Jones, K. A., Kadomaga, J. T., Luciw, P. A., and Tjian, R. (1986) Science 232, 755–759
20. Yedavalli, V. S. R. K., Benkirane, M., and Jeang, K.-T. (2003) J. Biol. Chem. 278, 6404–6410
21. Bieniasz, P. D., Grina, T. A., Bogerd, H. P., and Cullen, B. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7791–7796
22. Grignani, F., Mattei, S. D., Neri, C., Tomassoni, L., Gelfi, V., Cioce, M., Fanelli, M., Rutherford, M., Ferrara, F. F., Zinzari, I., Seiser, C., Grignani, F., Lazar, M. A., Minucci, S., and Pelicci, P. G. (1998) Nature 391, 815–818
23. Lin, R. J., Nagy, L., Inoue, S., Shao, W., Miller, W. H., and Evans, R. M. (1998) Nature 391, 811–814
24. Gea-Banacloche, J. C., and Lane, H. C. (1999) AIDS 13, (Suppl. A) S25–S38
25. Piersero, E., Teifel, T. L., Blankson, J., Finzi, D., Chadwick, K., Margolick, J. B., Buck, C., Siliciano, J. D., Doms, R. W., and Siliciano, R. F. (2000) J. Virol. 74, 7824–7833
26. Lescan, M. J., and Pendergrast, P. S. (1998) J. Biol. Chem. 273, 8125–8130
27. Lee, N. S., Dohjima, T., Bauer, G., Li, H., Li, M. J., Ehsani, A., Salvaterra, P., and Rossi, J. (2002) Nat. Biotechnol. 20, 509–505
28. Jacque, J. M., Friques, K., and Stevenson, M. (2002) Nature 418, 435–438
29. Wong-Staal, F., Poeschla, E. M., and Looney, D. J. (1998) Hum. Gene Ther. 9, 2497–2525
30. Amado, R. G., Mitsuyasu, R. T., Symonds, G., Rosenblatt, J. D., Zack, J., Sun, L. Q., Miller, M., Ely, J., and Gerlach, W. (1999) Hum. Gene Ther. 10, 2255–2270