JOURNAL OF VIROLOGY, Feb. 2004, p. 1456–1463 Vol. 78, No. 3
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.3.1456–1463.2004

Selection of TAR RNA-Binding Chameleon Peptides by Using a Retroviral Replication System

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Received 26 June 2003/Accepted 15 October 2003

The interaction between the arginine-rich motif (ARM) of the human immunodeficiency virus (HIV) Tat protein and TAR RNA is essential for Tat activation and viral replication. Two related lentiviruses, bovine immunodeficiency virus (BIV) and Jembrana disease virus (JDV), also require Tat ARM-TAR interactions to mediate activation, but the viruses have evolved different RNA-binding strategies. Interestingly, the JDV ARM can act as a "chameleon," adopting both the HIV and BIV TAR binding modes. To examine how RNA-protein interactions may evolve in a viral context and possibly to identify peptides that recognize HIV TAR in novel ways, we devised a retroviral system based on HIV replication to amplify and select for RNA binders. We constructed a combinatorial peptide library based on the BIV Tat ARM and identified peptides that, like the JDV Tat ARM, also function through HIV TAR, revealing unexpected sequence characteristics of an RNA-binding chameleon. The results suggest that a retroviral screening approach may help identify high-affinity TAR binders and may provide new insights into the evolution of RNA-protein interactions.

Many complex retroviruses, including the human immunodeficiency viruses (HIV) and related lentiviruses, encode RNA-binding proteins that regulate viral gene expression. While some RNA-binding domains may have been coopted from cellular genes, others may have arisen de novo and evolved in the viral context. The arginine-rich motif (ARM), found in several viral regulatory proteins, is a small (<20 amino acids) RNA-binding domain that is a particularly attractive candidate for the viral evolution model. It has been shown in several cases that short peptides corresponding only to the ARM bind RNA with high affinity and specificity, typically using few types of amino acids other than arginine to recognize their RNA sites (18, 38). In principle, the relative simplicity of the ARM might allow specific RNA-binding domains to evolve rapidly in the context of an error-prone retrovirus and potentially to coevolve with its RNA target. Studies with the HIV and related lentiviral Tat proteins, described below, support such a view.

HIV Tat is an essential viral transcription factor that enhances the processivity of RNA polymerase II by stimulating phosphorylation of its C-terminal domain (13, 19, 22, 23, 25, 36, 44, 53). Tat is brought to the HIV promoter by interacting with the TAR RNA hairpin located at the 5′ end of the viral mRNA transcripts and forms a ternary complex with cyclin T1, which recruits the Cdk9 kinase (5, 10, 20, 21, 27, 32, 59). Tat interacts with a 3-nucleotide bulge in TAR via a 9-amino-acid ARM (RKKRRQRRR) in which just one arginine (at position 52 of the protein) makes a base-specific contact (1, 9, 34, 42, 52, 57). Additional basic amino acids that surround this arginine enhance the affinity and kinetic stability of the complex and improve binding specificity (33, 34, 52, 58). Cyclin T1 further enhances Tat binding affinity and extends specificity to the 6-nucleotide loop, resulting in a functional complex (5, 10, 20, 21, 27, 32, 59).

The Tat proteins from two bovine lentiviruses, bovine immunodeficiency virus (BIV) and Jembrana disease virus (JDV), also use ARM domains to recognize their TAR sites, but in marked contrast to the HIV interaction, these ARMs adopt a β-hairpin conformation upon TAR binding and utilize several side chains other than arginine for specific RNA recognition (11, 12, 41, 49, 61). In these cases, cyclin T1 is not required to form stable, high-affinity RNA complexes and the loops do not contribute to recognition (2, 6, 11, 49). Despite the structural similarity of the TAR sites (50), BIV Tat binds HIV TAR only weakly and cannot use cyclin T1 as a cofactor to bind TAR. Interestingly, however, the JDV Tat ARM can act as a chameleon that binds RNA in two distinct binding modes, by using a cyclin T1-independent, β-hairpin conformation to bind BIV TAR and a cyclin T1-dependent, extended conformation to bind HIV TAR, utilizing one arginine for specific recognition (49). In the context of HIV-1 replication, hybrid viruses engineered with various HIV, BIV, and JDV Tat and TAR combinations have been shown to replicate only when high-affinity binding strategies are possible, including the chameleon behavior of the JDV ARM (60).

Given the sequence similarities between the HIV, BIV, and JDV Tat ARMs, the ability of the JDV ARM to bind to multiple RNA targets, and the observation that some TAR variants can bind both HIV and BIV Tat ARMs (50), it seems an ideal situation for new RNA-binding specificities to evolve. Indeed, when selective pressure was exerted on viral replication by engineering a noncognate Tat-TAR interaction into HIV-1, mutations readily arose that generated high-affinity interactions (60). Here we have constructed combinatorial peptide libraries within HIV-1 and have used viral replication to select for tight binders to HIV TAR. These experiments

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shed further light on the ability of RNA-peptide interactions to evolve within a viral context and on the differences in recognition strategies between the human and bovine Tat-TAR interactions. This selection strategy may be applicable to other type of RNA sites and may provide a means to identify inhibitors of RNA-protein interactions.

MATERIALS AND METHODS

Cell lines and viruses. Wild-type HIV-1 (from the R7/3 proviral plasmid) (17) and viral variants were cultured in 293T human embryonic kidney cells maintained in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin or in MT-4 cells maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS and 1% penicillin-streptomycin, as previously described (60). For chloramphenicol acetyltransferase (CAT) assay experiments, HeLa or NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1% penicillin-streptomycin.

Viral stocks were prepared by transfecting 10 μg of proviral plasmid DNA into 293T cells with calcium phosphate precipitation, collecting supernatants after 48 h, filtering through 0.45-μm-pore-size polyethersulfone membranes, and storing aliquots at −80°C. Levels of p24 capsid, measured by antigen capture enzyme-linked immunosorbent assay (Abbott Laboratories, Abbott Park, Ill.), were used to normalize virus levels for subsequent infection experiments. For infections, 10^6 MT-4 cells were infected with virus stocks (4 ng of p24) in 2 ml of RPMI 1640 medium at 37°C, particles were adsorbed for 2 h, cells were washed extensively to remove unadsorbed particles, additional medium was added (to 10 ml), and cultures were maintained by splitting (1:10) every 3 or 4 days. Viral titers and replication rates were monitored by p24 enzyme-linked immunosorbent assay and reverse transcriptase assays of cell-free culture supernatants.

Combinatorial peptide library design and screening. HIV-1 proviral clones previously engineered with heterologous Tat-TAR interactions (60) were used to generate proviruses with combinatorial peptide libraries fused to Tat. In these chimeric viruses, the second nucleotide (U) of the wild-type HIV-1 *tat* gene initiation codon was deleted and a synthetic version of HIV-1 Tat (residues 1 to 72) was inserted into the *nef* region, providing a functional Tat that supported replication (60) (also see Fig. 1B). In some chimeras, the HIV Tat ARM (residues 49 to 57) was replaced with the BIV Tat ARM (residues 65 to 81), resulting in replication-incompetent viruses when HIV TAR was present because the BIV ARM only weakly binds to HIV TAR (11). Here we generated viruses with combinatorial libraries based on the BIV Tat ARM in order to identify variants that bind HIV TAR. The encoded peptides contain five randomized positions within the BIV Tat ARM, XXXXXRGTRGKGRRIRR, where X represents any of 12 amino acids (see Results and Fig. 1C). A degenerate oligonucleotide (5′-CC CTA GGA ATC TCT TAC GGC XXY XXY XXY XXY XXY AGA GGT ACC AGA GGA AAG GGA AGG AGG ATC AGG AGA-3′, where X is a mixture of A:G:C [3:2.5:3] and Y is a mixture of G:T [2.5:3]), was synthesized encoding the arginine-rich peptide library. A second oligonucleotide (tat3, 5′-CGA GCT TAC GCG TCA CTG TTT AGA CAG AGA AAC CTG GTG GGT CTG CGA TCC CTG CGG CGG TCT CCT GAT CCT CCT TC-3′) was annealed to the degenerate oligonucleotide, double-stranded DNA was synthesized with Sequenase 2.0 (United States Biochemical), and the product was digested with *Hinf*I (underlined sequence in the degenerate oligonucleotide). A second pair of oligonucleotides (tat1, 5′-AAA CAC CCC GGG TCC CAG CCG AAA ACC GCG TGC ACC AAC TGC TAC TGC AAA AAA TGC TGC TTC-3′; and tat2, 5′-GCC GTA AGA GAT TCC TAG GGC TTT GGT GAT GAA GCA AAA CCT GGC AGT GGA AGC AGC ATT TTT TGC-3′) was annealed, double-stranded DNA was synthesized, and the product was digested with *Hinf*I (underlined sequence in tat2). The two *Hinf*I-digested fragments were ligated, digested with *Xma*I and *Mlu*I (underlined sequences in tat1 and tat3), and then ligated into an *Xma*I-*Mlu*I-digested HIV-1 proviral vector (pR7BtatHTAR [60]) to generate fusions to amino acid 48 of HIV Tat. The ligation mixture was electroporated into *Escherichia coli*, ~10^5 colonies were collected, and plasmid DNA was prepared with a Qiagen Mix kit.

To select for replication-competent viruses from the library, 10 μg of DNA was transfected into 293T cells, the supernatant was collected after 48 h and used to infect MT-4 cells, and viruses encoding binders were selected following multiple rounds of virus passage (see Fig. 1D). Peptide and TAR sequences were examined after the 3rd, 5th and 12th passages by PCR amplifying fragments from infected-cell genomic DNA with Tat primers (sense, 5′-GTA GCC AAG CTT ATG GAA CCG GTC-3′, and antisense, 5′-TTA CCG CTC GAG GAC GCG TCA CTG TTT AGA-3′; *Hin*dIII, *Xho*I, and *Mlu*I sites underlined) or TAR primers (sense, 5′-GAG AGC TGC ATC CGG AGT ACT TC-3′, and antisense, 5′-GCT CTA GAG CGG CCG CTA GAG ATT TTC CAC-3′), inserting the PCR products into a Topo vector (pCR2.0 Invitrogen, Carlsbad, Calif.), and dideoxynucleotide sequencing. To confirm that the selected peptide sequences alone were responsible for replication, *Xma*I-*Mlu*I-digested PCR fragments from above were recloned into pR7BtatHTAR and viral replication kinetics were measured.

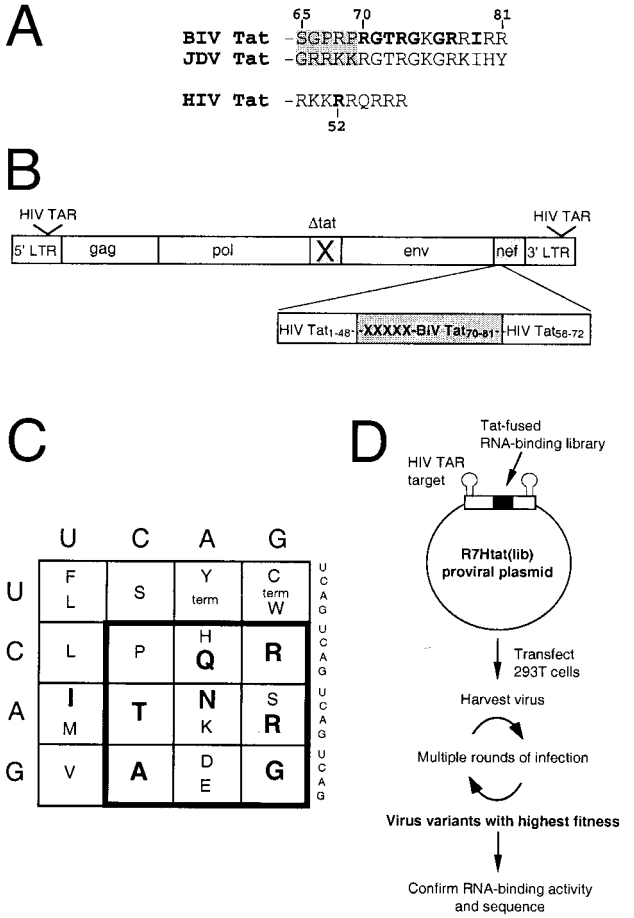

FIG. 1. (A) Sequences of the BIV, JDV, and HIV-1 Tat ARMs, aligned with respect to the amino acid similarity of their activation domains (not shown). The numbering refers to amino acid positions in their respective proteins, and residues highlighted in boldface are important for binding to their respective TAR sites. (B) Genomic arrangement of the HIV-1 proviral clone showing replacement of the HIV Tat ARM with the peptide library and the *tat* gene positioned to support virus replication (60). (C) The 12 amino acids used in the library are encoded by 18 codons, as indicated by the boldfaced box in the codon chart. (D) Strategy for isolating viral variants with high-affinity Tat-TAR interactions.

CAT reporter assays. Tat activation assays were performed with Tat fusion proteins containing the selected peptide sequences and HIV-1 long terminal repeat (LTR) CAT reporters containing HIV or BIV TAR sites (11, 49). Tat fusions were expressed from pSV2-derived vectors in which the HIV Tat activation domain (residues 1 to 48) was fused to the BIV ARM (residues 65 to 81) or selected peptide sequences, followed by HIV Tat residues 58 to 72. Fragments encoding peptide library members were obtained from *Xho*I-*Hin*dIII digests of the subcloned pCR2.0/Tat vectors described above and were inserted into an *Xho*I-*Hin*dIII-digested pSV2tat vector. For HeLa cell transfections, 100 ng of reporter plasmid and 50 ng of Tat-expressing plasmid were cotransfected with carrier plasmid (pBluescript DNA; total DNA was adjusted to 2 μg) by using 5 μl of Lipofectin (Life Technologies) in 3.8-cm^2 wells for 4 h. For NIH 3T3 cell transfections, 100 ng of reporter plasmid and 50 ng of Tat-expressing plasmid were cotransfected with carrier plasmid (pBluescript DNA; total DNA was

adjusted to 1 μg) by using 4 μl of Lipofectamine (Life Technologies) for 5 h. For some experiments in 3T3 cells, 100 ng of a plasmid expressing human cyclin T1 (residues 1 to 272) (59) was also cotransfected. Cell extracts were assayed for CAT activity after 48 h as described previously (11). Activities were quantitated with a Molecular Dynamics PhosphorImager, and activation (*n*-fold) relative to that for the reporter plasmid alone was calculated. For each experiment, CAT assays were performed in duplicate and percentages of activation for the different reporters or protein mutants relative to those for the wild-type combination were calculated. Percentages of activation were then averaged over three or four separate transfection experiments.

RNA-binding gel shift assays. Randomly labeled TAR RNAs were transcribed by T7 RNA polymerase by using synthetic oligonucleotide templates (37) and [^{32}P]CTP or UTP (3,000 Ci/mmol) and were purified on 15% polyacrylamide–8 M urea gels and were renatured as described earlier (49).

To examine ternary complex formation with cyclin T1 and various Tat proteins, GST-human cyclin T1 (1-272) was expressed and purified as described earlier (59). GST-Tat proteins (residues 1 to 72 of HIV-1 Tat containing various ARM substitutions) were expressed in *E. coli* BL21 cells and were purified on glutathione-Sepharose beads (Amersham-Pharmacia Biotech) by eluting with 50 mM reduced glutathione in 200 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Protein purity was assessed by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and concentrations were determined with Bradford assays (Bio-Rad, Hercules, Calif.). For gel shift assays, proteins (300 ng of GST-Tat and 1.6 μg of GST-cyclin T1, as indicated in Fig. 4) were incubated with RNA for 20 min at 30°C in 30 mM Tris-HCl, pH 8, 70 mM KCl, 0.01% NP-40, 5.5 mM $MgCl_2$, 1 mM dithiothreitol, and 12% glycerol (12-μl final volume). RNA-protein complexes were resolved on 6% polyacrylamide Tris-glycine gels at 4°C, and bands were visualized by phosphorimaging.

To examine peptide-RNA complexes, peptides were synthesized on an Applied Biosystems Model 432A peptide synthesizer by using 9-fluorenylmethoxy carbonyl chemistry and were purified by reverse-phase high-performance liquid chromatography as described earlier (49). The identity of the peptides was confirmed by matrix-assisted laser desorption ionization mass spectrometry, and peptide concentrations were determined by quantitative amino acid analysis. For gel shift assays, peptide and RNA were incubated together for 10 to 30 min on ice in 10-μl binding reaction mixtures containing 10 mM HEPES-KOH (pH 7.5), 100 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 50 μg of *E. coli* tRNA/ml as a competitor, and peptide-RNA complexes and free RNAs were resolved on 10% polyacrylamide—0.5× Tris-borate-EDTA gels at 4°C.

RESULTS

Selection of replication-competent HIV-1 variants to identify TAR-binding peptides. The HIV, BIV, and JDV Tat proteins display three different RNA-binding modes: HIV Tat utilizes an extended ARM conformation and requires cyclin T1 for high-affinity HIV TAR binding, the BIV ARM adopts a β-hairpin conformation and binds BIV TAR with high affinity in the absence of cyclin T1, and the JDV ARM is a "chameleon" that can bind to both HIV and BIV TARs in their respective binding modes (1, 9, 41, 42, 49, 61). The BIV ARM cannot bind HIV TAR in a cyclin-T1-dependent manner despite its similarity to the JDV ARM (see Fig. 1A), nor can HIV TAR be recognized by the β-hairpin conformations of BIV or JDV ARMs even though the bound structures of HIV and BIV TAR are nearly identical (50).

To further explore the requirements for chameleon-like binding and possibly to identify variant ARMs that can bind HIV TAR in a high-affinity, β-hairpin conformation, we generated a combinatorial peptide library in the context of an HIV-1 provirus (Fig. 1B) and used viral replication as a means to select HIV TAR binders from the population (Fig. 1D). Proviruses containing HIV TAR and the BIV Tat ARM are replication incompetent, whereas those with HIV TAR and the JDV Tat ARM replicate well (60). Thus, we designed the library with the BIV Tat β-hairpin core sequence (residues 70 to 81) and five randomized N-terminal residues that differ between BIV and JDV Tat (Fig. 1A), expecting that JDV-like binders would replicate. The randomized positions were restricted to 12 types of amino acids that include the hydrophilic or charged amino acids often used for nucleic acid interactions, as well as Ala, Gly, and Pro to provide conformational diversity to the peptides (Fig. 1C) (51). The library, which contains 18^5 ($\sim 1 \times 10^6$) codon sequences encoding 12^5 ($\sim 2 \times 10^5$) peptides, was fused to the HIV-1 Tat activation domain (residues 1 to 48), and proviral plasmid DNA was transfected into 293T cells. Supernatants containing replicating viruses were collected and passaged in MT-4 cells for 12 rounds. Extensive HIV-induced syncytia were observed 4 days after the initial round of infection (0.1 to 1.0 multiplicities of infection based on p24 levels from the transfection), indicating that a significant fraction of the library was replication competent.

TABLE 1. Sequences of selected TAR binders

Round of replication	No.	Sequence
3rd	3-1	**Q**RRRR
	3-2	GTRG**R**
	3-3	GAHA**R**
	3-4	ARPRR
	3-5	TRGQ**K**
	3-6	RRTRK
	3-7	ARNG**R**
	3-8	PRKRA
	3-9	RARH**R**
	3-10	TRGQ**K**
5th	5-1	SGKH**K**
	5-2	ARHT**R**
	5-3	RKRRQ
	5-4	RKRSA
	5-5	AKPRR
	5-6	GRRRN
	5-7	GAQ**RR**
	5-8	KRRGP
	5-9	GKQ**RR**
12th	12-1	RRRRP (3)[a]
	12-2	RKRRR (2)
	12-3	GKRRR (2)

[a] Numbers in parentheses refer to the number of times each clone was isolated.

Peptide sequences were determined following the 3rd, 5th, and 12th rounds of infection by using PCR to amplify *tat* fragments from integrated genomic DNA (Table 1). As described below in more detail, virtually all peptides had at least two positively charged residues within the randomized region, and by the 12th round, the "winning" sequences were highly enriched in arginine. To confirm that the PCR-derived sequences indeed encoded HIV TAR-binding peptides responsible for virus survival, we cloned 10 sequences back into the proviral plasmid and measured their individual replication rates (Fig. 2A). All showed replication rates approaching those observed with the HIV and JDV Tat ARMs, particularly for peptides isolated from the 12th round. To further confirm that the selected Tat proteins were active, we measured the activities of the proviral clones in HIV LTR CAT reporter assays that monitor Tat-mediated transcriptional activation. All showed activation levels comparable to proviruses containing the HIV or JDV Tat ARMs (Fig. 2B). The peptides from the "fittest"

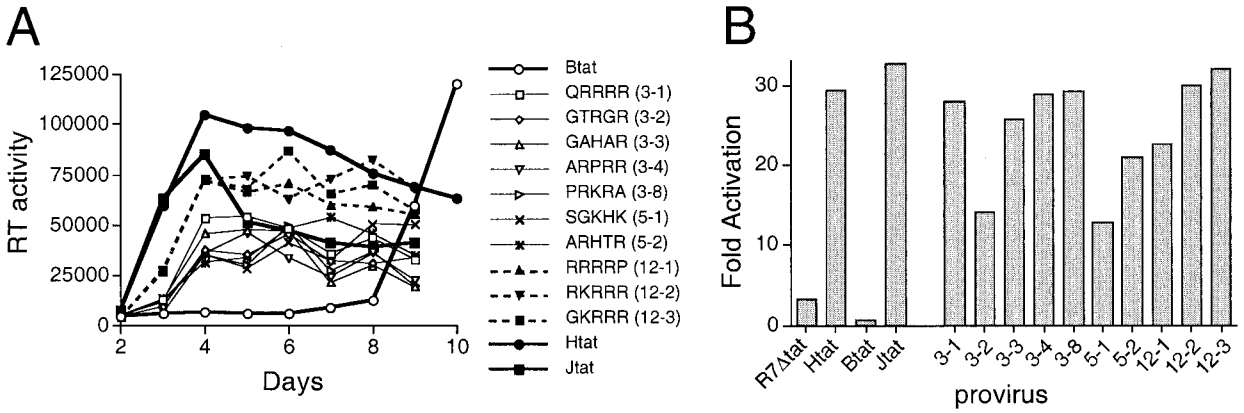

FIG. 2. (A) Replication kinetics of selected viruses grown in MT-4 cells. Htat, Btat, and JTat refer to viral clones containing HIV TAR and the indicated Tat ARM, as described previously (R7Htat, R7Btat-HTAR, and R7Jtat-HTAR) (60). Reverse transcriptase (RT) levels in the culture supernatants were measured at the times indicated. The high levels of virus observed with the Btat control after day 9 are presumed to reflect accumulation of a TAR mutation shown previously to generate a high-affinity binding site for the BIV Tat β-hairpin (60). (B) Transcriptional activation by the proviral plasmids. 293T cells were cotransfected with an HIV LTR CAT reporter plasmid and variant proviral plasmids, CAT activities were quantitated 48 h after transfection, and activation (*n*-fold) was calculated by dividing the amount of CAT activity in the presence of Tat by the amount in the absence of Tat. Data are representative of three independent experiments.

12th-round viruses contain three or more arginines, representing ~0.08% of the library sequences, and thus have been highly selected by viral replication.

HIV TAR binding mode of the selected peptides. To determine whether the selected ARM peptides adopt an extended conformation and require cyclin T1 when bound to HIV TAR, like the HIV and JDV ARMs, we cloned the proviral Tat-peptide fusions into a Tat expression vector and measured transcriptional activation with an HIV LTR CAT reporter. We measured activity in HeLa cells, which contain a cyclin T1 that supports HIV Tat activation, and in murine NIH 3T3 cells, which contain a cyclin T1 variant that cannot enhance the affinity and specificity of the Tat-TAR complex (5, 10, 20, 21, 32, 43, 59). All selected peptides activated transcription well in human cells (Fig. 3A) but poorly in mouse cells (Fig. 3B), indicating a weak affinity for HIV TAR in the absence of the Tat cofactor. Peptides 3-4 (ARPRR) and 12-2 (RKRRR) showed some activity on HIV TAR in 3T3 cells, perhaps suggesting a small degree of cyclin T1-independent binding.

To further confirm that cyclin T1 is required for the selected peptides to bind HIV TAR, we cotransfected human cyclin T1, known to complement the mouse cyclin (5, 10, 20, 21, 32, 43, 59), into 3T3 cells along with the Tat-peptide fusion proteins and measured activation levels. All proteins showed >5-fold-higher activities in the presence of cyclin T1 (compare Fig. 3B and C), consistent with the HIV binding mode.

To directly test whether the selected BIV Tat variants bind HIV TAR in a ternary complex with cyclin T1, we performed gel mobility shift assays with six different Tat proteins (containing the ARMs of BIV, HIV, JDV and the selected sequences 3-3, 3-4, and 12-2). Whereas the BIV Tat-HIV TAR complex did not form a ternary complex with cyclin T1 (Fig. 4, lane 3), all three selected sequences, as well as JDV and HIV Tat, efficiently recruited cyclin T1 to the RNA (Fig. 4, lanes 6, 9, 12, 15 and 18). Thus, the ability of the Tat variants to form ternary complexes in vitro correlates well with the cyclin T1-dependent activation observed in vivo.

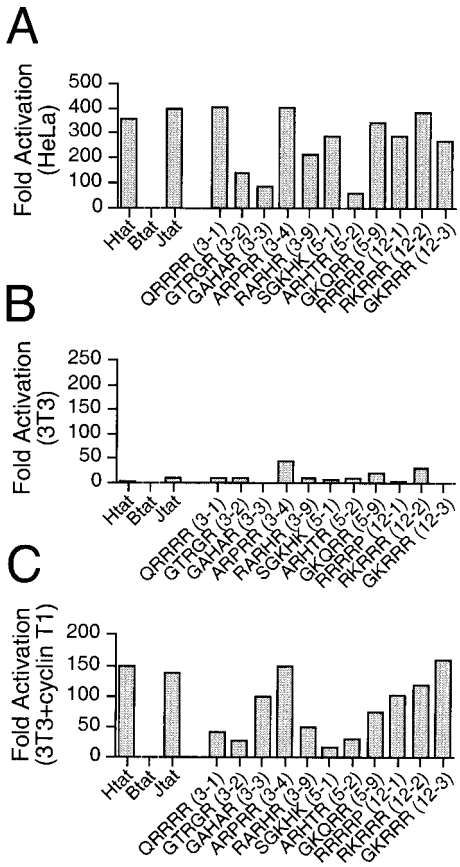

FIG. 3. Transcriptional activation through HIV TAR by the selected Tat fusion proteins. Tat expression plasmids were cotransfected into cells along with the HIV LTR CAT reporter, and activation levels were determined as described in the Fig. 2B legend. Activation levels are shown from transfections into HeLa cells, which contain a human cyclin T1 functional for Tat activity (A), murine NIH 3T3 cells, which do not contain a functional cyclin T1 (B), and murine NIH 3T3 cells cotransfected in addition with a human cyclin T1 expression plasmid (C).

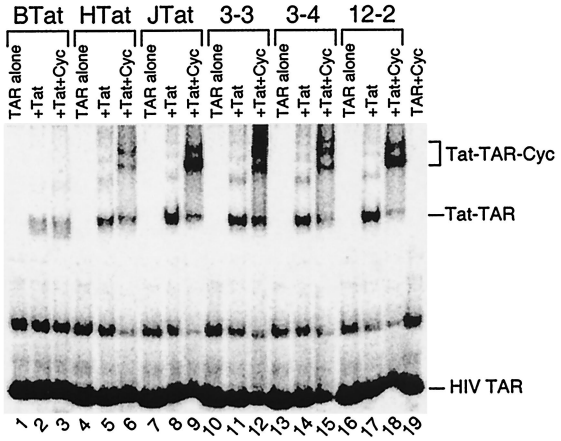

FIG. 4. Formation of ternary complexes between HIV TAR, human cyclin T1, and various Tat proteins. Radiolabeled HIV TAR RNA was incubated with 300 ng of GST-Tat proteins containing the ARMs of BIV Tat (BTat), HIV Tat (HTat), JDV Tat (JTat), or the selected sequences 3-3, 3-4, and 12-2 (lanes 2, 5, 8, 11, 14, and 17) or with the GST-Tat proteins and 1.6 μg of GST-human cyclin T1 (lanes 3, 6, 9, 12, 15, 18), and protein complexes were resolved by native gel electrophoresis. Control lanes with TAR alone (lanes 1, 4, 7, 10, 13, 16) and with TAR and cyclin T1 in which no complexes were observed (lane 19) also are shown.

Chameleon binding mode. To determine if the ARMs selected for HIV TAR binding retained their ability to bind BIV TAR, we measured transcriptional activation with an HIV LTR CAT reporter containing BIV TAR (11). All peptides (except 3-3, GAHAR; see below) activated well in both human and mouse cells (Fig. 5A and B), suggesting a high-affinity, cyclin T1-independent, β-hairpin binding mode. Thus, these peptides exhibit chameleon-like behavior similar to the JDV ARM, recognizing the two different TAR sites in their respective binding modes. Interestingly, only small sequence changes in the region N-terminal to the core BIV Tat ARM can generate a chameleon, with three changes sufficient to generate the 5-1 peptide (SG**KHK**) from BIV Tat (versus SG**PRP**).

Unexpectedly, one peptide (3-3; GAHAR) did not activate transcription through BIV TAR, even though all amino acids that participate in BIV TAR binding (between residues 70 and 79; Fig. 1A) are preserved in this construct. To test whether this particular sequence may have disrupted binding of the β-hairpin, we compared the BIV TAR binding affinities of synthetic GAHAR and BIV Tat peptides by in vitro gel shift assays. Both displayed similar binding affinities (Fig. 5C), suggesting that some sequences may provide an incompatible linkage to the Tat activation domain that interferes with RNA binding or transcriptional activation in vivo. The GAHAR sequence supports activity on HIV TAR, suggesting that the incompatibility is related to the β-hairpin binding mode.

Charge requirements for HIV TAR recognition. The sequences of the selected peptides (Table 1) suggested that two or more positively charged residues are needed in the N-terminal region of the BIV ARM (residues 65 to 69) to allow binding to HIV TAR and thereby act as a chameleon. We previously found that one arginine in the JDV ARM (at position 70) is critical for HIV TAR recognition, like in HIV Tat (49). Because three positively charged residues N terminal to the arginine are required for HIV TAR recognition (9, 52), we

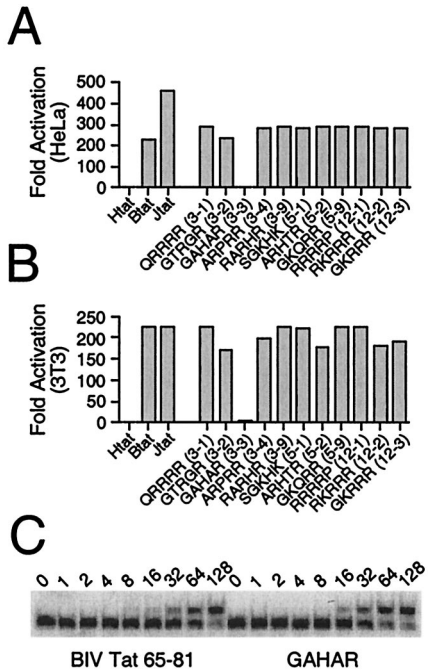

FIG. 5. Transcriptional activation through BIV TAR by the selected Tat fusion proteins and binding of the GAHAR peptide. Tat expression plasmids were cotransfected into cells along with an HIV LTR CAT reporter containing the BIV TAR element, and activation levels were determined as described in the Fig. 2B legend. Activation levels are shown from transfections into HeLa cells, which contain a human cyclin T1 functional for Tat activity (A), and murine NIH 3T3 cells, which do not contain a functional cyclin T1 (B). (C) RNA-binding gel shift assays with BIV TAR and BIV Tat 65-81 and GAHAR peptides. Binding reactions were performed at the peptide concentrations indicated in nanomolars.

presumed that the four basic residues preceding Arg70 in the JDV ARM contributed to its chameleon behavior, unlike the BIV ARM, which has only one basic residue in this region (Fig. 1A). To test the importance of basic residues in a selected peptide, we replaced Lys69 in the 5-1 peptide (SGKH**K**), whose sequence is similar to the BIV ARM (SGPRP), with Ala or Pro, and observed substantially reduced activities on the HIV TAR reporter (Fig. 6). Conversely, replacing either Pro67

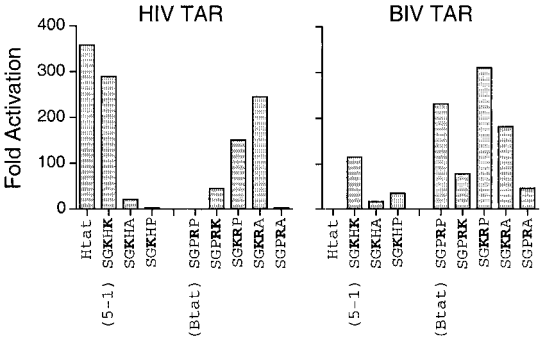

FIG. 6. Activities of BIV Tat ARM and selected peptide mutants with basic amino acid substitutions. Tat expression plasmids were cotransfected into HeLa cells along with HIV LTR CAT reporters containing HIV TAR (left panel) or BIV TAR (right panel) elements, and activation levels were determined as described in the Fig. 2B legend.

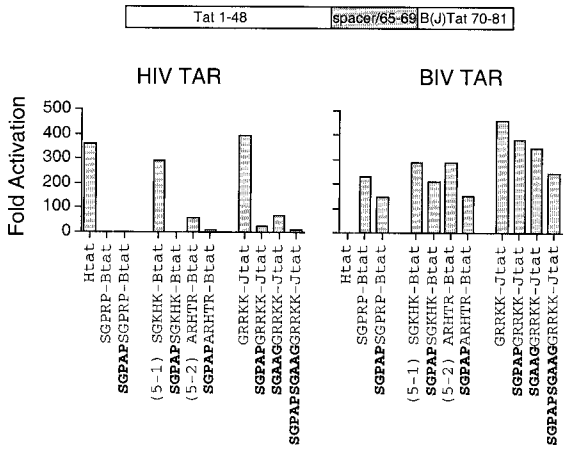

FIG. 7. Activities of peptide variants engineered with lengthened spacers between the Tat activation domain and ARMs. The schematic (top) indicates that the Tat fusions were constructed with variable spacers (shown as the boldfaced sequences on the *x* axis) following the HIV Tat activation domain, followed by residues 65 to 69 of BIV Tat, JDV Tat, or the library as indicated by the sequences, followed by the core β-hairpin domain of BIV or JDV Tat. Tat expression plasmids were cotransfected into HeLa cells along with HIV LTR CAT reporters containing HIV TAR (left panel) or BIV TAR (right panel) elements, and activation levels were determined as described in the Fig. 2B legend.

or Pro69 of the BIV ARM with Lys substantially increased activity (Fig. 6). To test whether activity of the BIV ARM might have increased because Pro residues were removed, we also replaced Pro69 with Ala and in this case observed no enhancement of activity (Fig. 6). In contrast, on the BIV TAR reporter, replacement of Pro69 decreased activity even when changed to a basic amino acid. Preliminary modeling suggests that Pro69 may help the β-hairpin avoid steric clashes with the terminal BIV TAR loop (V. Calabro, unpublished data), consistent with the observation that a hybrid peptide composed of the BIV N-terminal region and the JDV C-terminal region has enhanced affinity for BIV TAR (49). Thus, the sequence of the JDV chameleon has not been optimized for binding to both TARs, but it might evolve if selective pressure were simultaneously maintained for the two. Other selective influences on ARM function may occur in the context of the virus, including requirements for nuclear localization (14, 24, 47, 48) and post-translational modification such as Lys acetylation (7, 8, 16).

Length and sequence of the N-terminal region affect activity. Extensive studies of HIV Tat have established that the spacing between the activation domain (residues 1 to 48) and RNA-binding domain (residues 49 to 57, and Arg52 in particular) is critical for binding to HIV TAR, presumably because it helps define the orientation of cyclin T1 bound to the activation domain and interactions with the TAR loop (3, 35, 45, 62). In contrast, it is expected that altering the spacing between the activation domain and the BIV Tat ARM, or a chameleon ARM, would not affect activity on BIV TAR, given that binding is cyclin T1 independent. Thus, it should be possible to distinguish the β-hairpin binding mode from the extended cyclin T1-dependent binding mode by examining the sensitivity to spacing. Indeed, adding a 5-amino-acid spacer (SGPAP) between the HIV activation domain and either JDV Tat or two of the selected chameleon ARMs (5-1 or 5-2) sharply reduced activity on HIV TAR but had little effect on BIV TAR (Fig. 7). Adding a slightly different spacer sequence (SGAAG), which eliminated prolines that might also have influenced the peptide conformation, or adding a longer, 10-amino-acid spacer to the JDV domain yielded similar results (Fig. 7).

Given that spacer length and sequence affect chameleon ARM binding activity, we also wished to determine the effect of moving the core β-hairpin domain closer to the activation domain, particularly for BIV TAR, which in principle should be insensitive to the change. We generated a series of deletions by utilizing a BIV/JDV Tat hybrid ARM composed of the N-terminal BIV region (residues 65 to 69), which has only one basic amino acid and consequently should be inactive on HIV TAR, and the JDV core (residues 70 to 81), which binds BIV TAR with high affinity (49). Unexpectedly, activity decreased sharply on BIV TAR when the core β-hairpin was moved 2 amino acids closer to the activation domain (see SGP, SG, and S spacers; Fig. 8), but activity was highly sensitive to the amino acid sequence of the spacer (compare SGP to SGR, and SG to RP; Fig. 8). As expected, activity on HIV TAR was weak at any spacer length because the BIV/JDV hybrid ARM does not contain the required two N-terminal basic amino acids, whereas replacing Pro with a basic residue in the context of a 4-amino-acid spacer (SG**P**R to SG**R**R; Fig. 8) restored activity. Unexpectedly, however, just one basic amino acid was sufficient for activity on HIV TAR when the core β-hairpin was located two

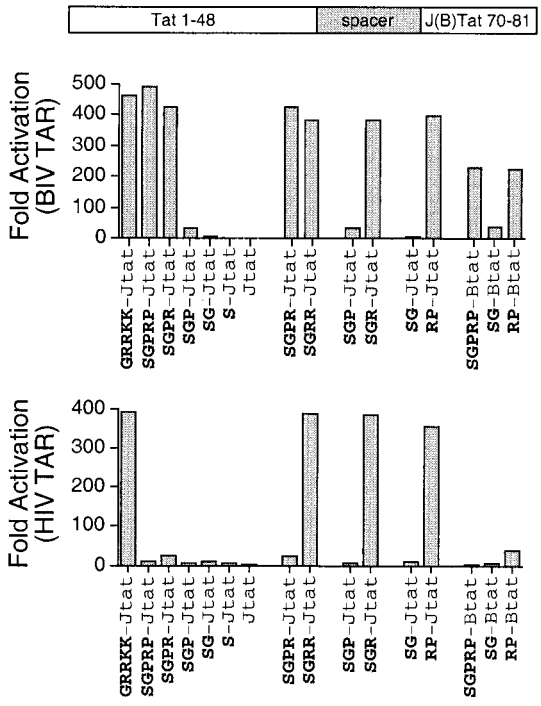

FIG. 8. Activities of peptide variants engineered with shortened spacers between the Tat activation domain and ARMs. The schematic (top) indicates that the Tat fusions were constructed with variable spacers (shown as the boldfaced sequences on the *x* axis) following the HIV Tat activation domain, followed by the core β-hairpin domain of BIV or JDV Tat as indicated. Tat expression plasmids were cotransfected into HeLa cells along with HIV LTR CAT reporters containing BIV TAR (upper panel) or HIV TAR (lower panel) elements, and activation levels were determined as described in the Fig. 2B legend.

or three residues from the activation domain (compare SG**P** to SG**R** and **S**G to **R**P; Fig. 8), thus generating two new chameleon ARMs. Even the BIV Tat core can function as a chameleon with the RP spacer, albeit more weakly than with the JDV core (Fig. 8). For all these chameleons, locating the core closer to the activation domains likely shifts the arginine used to make the sequence-specific HIV TAR contact from Arg70 (used by the JDV Tat ARM [49]) to Arg73, providing a new binding register with the appropriate number of surrounding basic residues (52). These spacing experiments further underscore the complexities of maintaining two types of binding modes within a single ARM and suggest that even more pathways may exist to evolving specific binding peptides via multifunctional intermediates than previously imagined (49, 50).

DISCUSSION

We have described a retroviral replication system to select RNA-binding peptides from combinatorial libraries and have identified ARM peptides that bind HIV TAR with chameleon-like characteristics similar to the JDV Tat ARM. The results indicate that the BIV ARM minimally requires two basic amino acids in its N-terminal region to recognize HIV TAR in a cyclin T1-dependent manner, although the fittest binders, as judged by virus replication rates, are highly enriched in basic amino acids, like the JDV ARM. Furthermore, by altering the spacing between the core β-hairpin and the Tat activation domain, we found yet another chameleon arrangement that appears to use a shifted binding register to contact HIV TAR. Thus, arginine-rich peptides may provide a particularly good framework to extensively sample sequence space while simultaneously maintaining more than one binding mode, thereby allowing both the RNA elements and RNA-binding domains to coevolve (49, 50). Comparisons between the HIV, BIV, and JDV Tat-TAR interactions have revealed multiple possible evolutionary pathways between the viruses, involving distinct peptide and RNA structures and cellular protein requirements (49).

The selective pressure exerted by retroviral replication, and in particular the requirement for the Tat-TAR interaction within lentiviruses, provides an excellent environment for peptide-RNA evolution. The importance of Tat function for viral fitness has been demonstrated by numerous studies of HIV-1 replication, and it has been estimated that ~15% of wild-type Tat activity is required to achieve measurable virus survival in tissue culture (56). When selective pressure was applied to HIV-1 replication either by introducing Tat (39, 55) or TAR (4, 31) mutations, revertants or compensatory mutations rapidly arose in the population. Similarly, when selective pressure was applied to HIV-1 by replacing the HIV-1 Tat ARM with the BIV Tat ARM, TAR variants arose that were able to bind the BIV Tat β-hairpin (60). In our present study with the BIV Tat ARM, no such TAR variants arose since the combinatorial library already contained a reasonable number of HIV TAR binders, thus placing little selective pressure on TAR. Comparisons between the Tat proteins of different viral clades show significant variation in their transcriptional activities (see reference 46), perhaps contributing to the evolution of viral subtypes under broader selective conditions.

Our experiments identified ARM sequences that bind HIV TAR and have high transcriptional activity, but other selective pressures undoubtedly influenced the outcome, as with any selection experiment. For example, it has been reported that acetylation of Lys50 in the ARM is important for synergistic activation with p300 (8, 30, 40), and indeed, several viruses isolated after 12 rounds of replication contained Lys at this position (Table 1). However, this was not exclusively the case, and the presence of Lys did not correlate with high transcriptional activity (Fig. 3), suggesting that acetylation of Lys50 is not essential for Tat activation in the proviral context or cell types utilized here.

Previous studies of HIV-1 variants engineered with heterologous Tat-TAR interactions within an otherwise identical virus background demonstrate that rates of viral replication correlate well with RNA-binding affinities (60). Those studies, and the results presented here, suggest that viral selection may be employed to identify high-affinity TAR-binding peptides. By engineering other combinatorial libraries based on the BIV and JDV Tat ARMs, including spacers that do not allow a chameleon binding mode, it may be possible to identify β-hairpin binders to HIV-1 TAR that can effectively displace the Tat-cyclin T1-TAR complex. Furthermore, it may be possible to identify binders to other RNA sites (ARM peptides or other types of domains) using appropriately TAR-substituted viruses that still maintain the other reported functions of TAR (15, 26, 28, 29, 54). By engineering diverse viral populations with combinatorial libraries, it should be possible to rapidly select for the fittest viral variants.

ACKNOWLEDGMENTS

We thank Chandreyee Das, Steve Landt, and members of the Frankel laboratory for helpful discussions and Mervi Detorio and Maureen Oliveira of the Wainberg laboratory for technical assistance.

This research was supported by NIH grant RO1 AI29135 (A.D.F.) and by the Canadian Institutes of Health Research (M.A.W.).

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