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A Selection System to Study Protein–RNA Interactions: Functional Display of HIV-1 Tat Protein on Filamentous Bacteriophage M13

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The transactivator protein (Tat) of the human immunodeficiency virus (HIV) is a key regulatory protein in the viral replication cycle and belongs to the RNA binding proteins of the arginine-rich motif (ARM) family. Very little is known about their mechanism of RNA recognition. To study the principles of RNA-protein recognition we constructed a system to display HIV-1 Tat on the surface of the filamentous bacteriophage M13. HIV-1 Tat (1-72) and a mutant Tat lacking five cysteine residues were cloned into the pAK phagemid system, which allows fusion of the *tat* gene to a supershort version of the gene for minor M13 coat protein. Expression of the resulting fusion proteins was shown via western blot analysis. Phages displaying functional Tat could be selected from phages without Tat or with a non-functional Tat variant via binding to biotinylated TAR using streptavidin coated paramagnetic beads. By randomizing certain amino acid positions of Tat and screening of the resulting phage libraries for affinity and specificity, we are now able to study the role and importance of amino acids of HIV-1 Tat for affinity and specificity to TAR RNA.

RNA-binding proteins play key roles in the regulation of eukaryotic gene expression. Viral strategies for genetic control of their host cells often depend on the action of RNA-binding proteins. Thus, it is of utmost importance to understand the mode of protein-RNA recognition. Most of the RNA-binding proteins can be divided into classes depending on the presence of amino acid sequence motifs (1).

Few protein-RNA complex structures are known so far. Even from the few published high resolution structures in the Brookhaven data base it is impossible to deduce general rules for specific recognition of RNA by polypeptides. A more comprehensive way to study protein-RNA recognition requires a firm basis of mutational data in addition to structural data.

In the past few years technologies have been developed and successfully applied, that allow screening of synthetic polymer pools with large, and biopolymer pools with very large diversity (2,3). A commonly used technique is the display of peptide libraries on the surface of filamentous bacteriophages (4). Sufficiently displayed and physically accessible, these peptides can act as ligands or substrates and be screened for suitable properties in a variety of biologically important systems, such as antibodies (5), hormone-receptors (6), enzymes (7,8) and nucleic acids (9,10).

Our intention is to study the role of amino acid residues for affinity and specificity to RNA in a model system, the human immunodeficiency virus type 1 (HIV-1) transactivator protein (Tat). The replication of lentiviruses depends on the action of this potent transactivator. Therefore, lentiviral Tat proteins, especially that of HIV-1, have been subject to extensive investigations. It is known that Tat binds to an RNA element (TAR, transactivator responsive element) located at the 5′-end of all viral transcripts. Upon its formation the Tat-TAR-complex enhances transcription of all viral genes (11). The first exon of *tat* is completely sufficient for all known biological activities, except the intercellular exchange in cell culture (12,13). HIV-1 Tat, like other lentiviral Tat proteins, can be divided into an N-terminal, cysteine-rich, core, basic and glutamine-rich sequence region (Fig. 1A). The basic and proximal parts of the core and glutamine-rich sequence regions are responsible for protein-RNA interaction (14). The basic sequence region contains many arginine residues, and hence, Tat is included in the arginine-rich motif (ARM) family of RNA binding proteins.

Binding of HIV-1 Tat to TAR is currently studied by a variety of methods. Tat binding to TAR was shown to be extremely specific and tight. The dissociation constant (k_D) is in the nanomolar range (14) or even below

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(15). The structures of the HIV-1 Tat and the closely related Tat of the equine infectious anemia virus (EIAV) have been determined (16,17). Structural studies of HIV-1 Tat peptide-TAR complexes (15,18-22) yielded substantial information about the structure of the RNA part of the complex, but yielded for the peptide part only adoption of an extended conformation. From these and structural studies on the binding of bovine immunodeficiency virus (BIV) Tat peptides to BIV TAR RNA (23,24) one can conclude that HIV-1 and BIV Tat proteins bind to a bulge region in the overall stem loop structure of the respective RNA elements.

The ARM seems to adopt completely different secondary structures depending on the respective protein. In HIV-1 and BIV Tat it adopts an extended β-sheet-like structure, whereas in bacteriophage Lambda N-protein, HIV-1 Rev (regulator of virion expression) and EIAV Tat it seems to adopt an α-helical secondary structure (17,25-27). Functional interchangeability of several ARM domains was investigated by an *in vivo*-selection procedure (28). However, with this system no positive functional results were obtained for HIV-1 Tat.

Another system was already used earlier to study protein-RNA interactions by *in vitro*-selection techniques. There, an RNP-domain of the mammalian spliceosomal protein U1A was locally mutagenized and displayed as a combinatorial library on filamentous bacteriophage. Affinity selection identified four amino acid residues in the mutagenized region that are important for specific binding to U1 hairpin II (10). For the RNA moiety of some protein-RNA complexes, mutational data was derived from SELEX procedures (29-32).

As a prerequisite to study the role of amino acid residues in HIV-1 Tat for affinity and specificity to TAR RNA, we constructed a phagemid system that displays Tat as fusion protein with a supershort version of M13 minor coat protein, Gp3ss. In the present work we demonstrate correct expression of the fusion peptide, as well as display of functional Tat on the phage surface. Because it is known that cysteines can interfere with incorporation of the Gp3-fusion protein into the phage particle (33,34), it was necessary to investigate whether an extreme cysteine-rich protein like HIV-1 Tat with an odd number of cysteines is incorporated into the phage coat or a mutant Tat lacking the five cysteines of the cysteine-rich sequence region is incorporated to a higher extent.

MATERIALS AND METHODS

Antibodies used. Monoclonal mouse anti-Gp3 antibodies were purchased from MoBiTec (Göttingen, Germany). Rabbit anti-mouse IgG-alkaline phosphatase conjugates were from DAKO (Glostrup, Denmark). Both antibodies were used at a 1:1000 dilution.

Oligonucleotides. PAK/NOTAT, CCTGCTATCGATGACCTTC (19mer); PAKSEQ/GP3, CCCTTATTAGCGTTTGCC (18mer); PAKSEQ/PELB, CCTATTGCCTACGGCAGC (18mer); PAKPELB/TAT, GGAAAGGCCCAGCCGGCCATGGCGCTCGAGCCGGTCGACCGGAACATC (48mer); PAKGP3/TAT ACCTTAGGCCCCCGAGGCCTGTTTCGGGATCGGGTCTTGG (40mer); PAK/TAT/ALA5′ GCGCTCGGAATTTCTTACGGCCGTAAGAAACGTGCGCAGGCGGCCGCGCCGTCTCAGGGTGGTCAAACTCATCAG (75mer); PAK/TAT/ALA3′ GTCCTGATGAGTTTGACCACCCTGAGACGGCGCGGCCGCCTGCGCACGTTTCTTACGGCCGTAAGAAATTCCGAG (75mer); PAK/E1095′ CACTAAGGGCCTCGGAATTTCTTACGGCC (29mer); PAK/E1093′ GCCCTCGGCCCCCGAGGCCTGTTTCGGGATCGGGTCCTGATGAGTTTGACCACC (54mer, all purchased from MWG Biotech, Ebersberg, Germany).

Plasmids used. Phagemid pAK200 (35) was kindly provided by Prof. A. Plückthun, Universität Zürich, Switzerland. The HIV-1 Tat expression vector pTK1 (36) was kindly provided by Dr. Thomas Kirsch and Prof. P. Rösch, Universität Bayreuth, Germany. Plasmid pTK72mt5cys codes for mutant Tat lacking the five cysteines of the cysteine-rich sequence (C22S, C25A, C27K, K29D, C30P, C31S) (Silke Hoffmann, personal communication).

DNA sequence analysis. DNA sequences were determined using GATC-BioCycle sequencing kit, GATC 1500 system (GATC, Konstanz, Germany) and Thermo Sequenase (Amersham, Cleveland, Ohio).

Construction of phagemids pAK-tat-wt, pAK-tat-mt5cys, and pAK-tat-mt4arg. The part of the synthetic *tat* gene of pTK1 (36) equivalent to the fully biological active first exon of HIV-1 *tat* was amplified by polymerase chain reaction (GeneAmp 2400, Perkin Elmer, Norwalk, CT, USA) employing 50 pmol of oligonucleotides PAKPELB/TAT and PAKGP3/TAT, 100 ng pTK1 and 3 U Vent-DNA polymerase (New England Biolabs) in 50 μl 1 $\times$ Vent-polymerase buffer containing 200 μM dNTP's and 4 mM $MgSO_4$. The second codon of Tat GAC (Asp) was replaced by GAG (Glu) resulting in an additional restriction site for *Xho*I. pAK-tat-mt5cys was constructed analog to pAK-tat-wt using pTK72mt5cys instead of pTK1 as template. PCR products were separated by electrophoresis in 1.5 % (by weight) agarose gels and extracted (Qiagen gel extraction kit). The resulting *tat* gene flanked by *Sfi*I recognition sites was restricted with *Sfi*I (Eurogentec, Seraing, Belgium), at 55°C for 3 hours in 40 μl 10 mM Tris/HCl (pH 7.5, 35°C), 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA. The resulting product was ligated with *Sfi*I restricted dephosphorylated pAK200. Ligation product was electroporated (E.coli Pulser, Biorad, München, Germany) into *E.coli* XL1 Blue cells (Stratagene) (37). Positive transformands were DNA sequenced. pAK-tat-mt4arg was constructed from pAK-tat-wt by cassette mutagenesis introducing annealed oligonucleotides PAK/TAT/ALA5′ and PAK/TAT/ALA3′ between two non-equivalent *Eco*O109I sites, that were generated in pAK-tat-wt by PCR mutagenesis (38) employing oligonucleotides PAK/E1095′, PAK/E1093′ and PAKSEQ/PELB. DNA sequence was verified after each step.

Phage growth. *E.coli* XL1 Blue cells harboring pAK-tat-wt, pAK-tat-mt5cys, pAK-tat-mt4arg or pAK200 were grown at 37 °C in 50 ml medium containing 12.8 g/l trypton, 8 g/l yeast extract, 4 g/l NaCl, 10 g/l glucose, 30 mg/l chloramphenicol, 10 mM sodium phosphate buffer, pH 7.0. At an optical density at 600 nm of 0.6 VCS-M13 helper phages (Stratagene) were added to an moi of 20. After 15 minutes 100 ml medium and 1 mM IPTG (end concentration) were added to induce expression of Gp3ss fusion protein. After two hours 30 μg/ml kanamycin (end concentration) were added and cells were grown at 30 °C for 12 hours. Phages were harvested by centrifugation (2 $\times$ 15 min, 8000 $\times$ g) of the cultures and subsequent precipitation of phages from the remaining supernatant by adding 4 % (by weight) polyethylenglycole (PEG 8000) and 0.5 M NaCl (end concentrations) by centrifugation (20 min, 16000 $\times$ g) after 15 min incubation on ice.

Western blot analysis. Samples of about 10^{11} phage particles were separated by a denaturing 15 % SDS-polyacrylamide gel electrophoresis, electrophoretically transferred to a nitrocellulose membrane

and incubated with a monoclonal mouse anti-Gp3 antibody (MoBiTec, Göttingen, Germany) at a dilution of 1:1000. Gp3-anti-Gp3 antibody complexes were detected by treatment of the filter with rabbit anti-mouse IgG-alkaline phosphatase conjugate (DAKO, Glostrup, Denmark; 1:1000 dilution) and subsequent development with nitrotetrazolium blue and 5-bromo-4-chloro-indolylphosphate. Molecular weight standard proteins (S) were separated and blotted together with phage proteins but stained with Ponceau S (Merck, Darmstadt).

PCR product length analysis. Phagemid harboring *E.coli* XL1 Blue colonies were picked from agar-plates (LB, 30 mg/l chloramphenicol, 1 % glucose) using a sterile toothpick and were transferred to 0.2 ml PCR-reactions containing standard buffers, Taq-polymerase (MBI Fermentas, Vilnius, Lithuania) and three primers (PAKSEQ/PELB, PAK/NOTAT, PAKSEQ/GP3), which are complementary to the *pel*B-, *tet*-spacer, and *gp3*-sequence, respectively. PCR products obtained with a very short extension time (25 s) were separated on 3 % agarose gels. Amplificates with a length of 287 bp correspond to pAK200 and those with 345 bp correspond to pAK-wt-tat.

Restriction assay analysis. Plasmid preperations from mixtures of pAK-tat-wt and pAK-tat-mt4arg were restricted with *Not*I. Complete restriction was verified by doing control experiments with pure pAK-tat-wt and pAK-tat-mt4arg DNA, of which only latter contained a *Not*I restriction site. Resulting DNA pieces were seperated by agarose gel electrophoresis. Bands corresponding to either pAK-tat-wt or pAK-tat-mt4arg were identified from the control experiments and quantitated densitometrically.

Selection procedure. For every round of selection, 50 μl binding reaction containing about 10^{11} phage particles, 25 ng (rounds 1 to 3) or 250 ng (round 4) TAR-RNA (5′-biotinylated), 2.5 μg tRNA, 1 mM DTT and 40 U RNasin in 100 mM NaCl and 10 mM Tris-HCl, pH 6,9 was incubated for 30 min at room temperature. The binding reaction was added to 0.7 mg streptavidin coated beads (CPG, Lincoln Park, NJ) for further 30 min. The beads were washed sixteen times with 200 μl 100 mM NaCl, 10 mM Tris-HCl, pH 6,9, 1 % dried milk powder, 0,5 % Tween 20 and three times with 200 μl 100 mM NaCl, 10 mM Tris-HCl, pH 6,9. Bound phages were eluted by addition of 0,1 M triethylamine for 5 minutes and subsequent neutralization with an equal volume of 1 M Tris-HCl, pH 8,0. The eluted phages were used to infect *E. coli* XL1 Blue cells to generate colonies of pAK harboring cells. Phages were grown as described above.

RESULTS AND DISCUSSION

HIV-1 Tat is one of the most thoroughly studied RNA binding proteins. But especially *in vitro*-studies are sig-

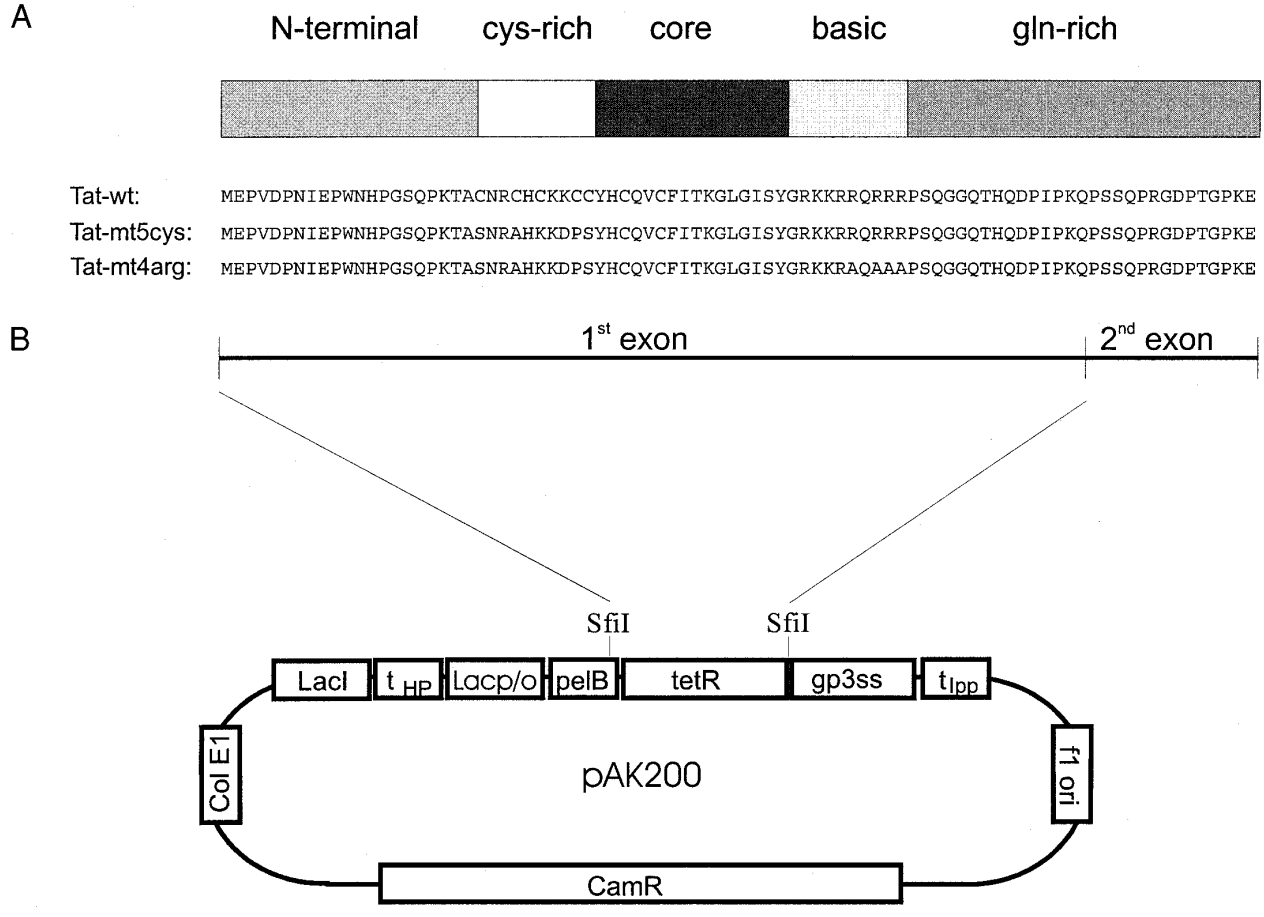

FIG. 1. Sequence domain structure of HIV-1 Tat and construction of the phagemid vector. (A) Sequences of wild type Tat (Tat-wt) and variants of Tat lacking the five cysteines of the cysteine-rich sequence (C22S, C25A, C27K, K29D, C30P, C31S; Tat-mt5cys) or lacking four arginines of the basic sequence (R53A, R55A, R56A, R57A; Tat-mt4arg). (B) Construction of the phagemid vector pAK-tat-wt. The part of the synthetic *tat* gene of pTK1 (36) equivalent to the fully biological active first exon of HIV-1 *tat* was inserted into pAK200 (35) replacing the *tet* spacer, which contains the *tet* gene and flanking sequences.

nificantly hindered by its high and odd number of cysteine residues (seven cysteines within sixteen residues, Fig. 1A). It is known that cysteines in peptides that are to be displayed on phages, can interfere with incorporation of the Gp3-fusion protein into phage particles (34), especially if the number of cysteines is odd (33). Therefore, the synthetic gene coding for *tat* was taken from plasmid pTK1 (36) and introduced into the phagemid vector pAK200 (35) (Fig. 1B), which allows fusion of *tat* to a supershort version of the *gp3* gene (*gp3*ss). Further, a mutant of *tat* (mt-*tat*) lacking the five cysteines of the cysteine rich region was constructed and introduced into pAK200. The sequences of the resulting phagemids pAK-wt-tat and pAK-mt-tat, respectively, were verified by DNA sequence analysis (data not shown).

An additional important property of pAK200 is minimization of undesired background expression of the Gp3ss-fusion protein with an additional strong terminator between the *lac* repressor gene and the *lac* promoter/operator sequence (39). We were not able to express a *tat-gp3* fusion in less tight regulated phage display vectors, because the toxicity of the Tat-Gp3 fusion protein leads to high frequency of undesired recombination events during the selection procedure, resulting in genetic instability of such vector systems (data not shown).

To test whether wt-*tat-gp3ss* and the mt-*tat-gp3ss* fusions are expressed and incorporated into phage particles, bacteria harboring pAK-tat-wt or pAK-tat-mt5cys were superinfected with helper phage VCS-M13. During phage production, expression of fusion protein was induced with IPTG. The supernatants containing helper and phagemid particles were analyzed for the presence of phagemid encoded Gp3 fusion protein by western blot analysis using anti-Gp3 antibodies (Fig. 2).

The result shows immunostaining of a protein that migrates with an apparent molecular weight of about 67 kDa, corresponding to helper phage encoded full length Gp3. In lines 3 and 4 an additional band equivalent to a molecular weight of about 25 kDa can be identified. This corresponds to correct expression and incorporation of wt-Tat-Gp3ss and mt-Tat-Gp3ss fusion proteins, respectively, into phage particles. Densitometrically, the amount of Tat-Gp3ss incorporated into capsids could be determined to be about 20 % (by mol) of wt-Gp3. This leads to the suggestion that one Tat molecule in average is presented per capsid and that the cysteine-rich region of Tat does not influence incorporation of wt-Tat-Gp3 fusion protein into phages.

Correct assembly of HIV-1 Tat into capsids does not necessarily mean that the protein is also fully active in the sense of its affinity and specificity to TAR RNA. This can be easily tested in a simplified biopanning procedure that addresses the question whether Tat displaying phages can be reselected and enriched from a large excess of phages not displaying Tat on their surface. Thus, pAK-wt-tat and pAK200 phages were mixed at ratios of 1:5000 and 1:50000. To the mixtures, biotinylated TAR RNA and 100-fold excess (by weight) tRNA was added together with streptavidin coated magnetic beads. The magnetic beads then were separated from the remaining phage suspension and thoroughly washed. Bound phages were eluted by hydrolyzing TAR RNA. That way, phages could safely be disconnected from the magnetic beads without taking the risk of loosing the most tightly bound ones. These phages were then added to *E.coli* XL1 Blue. At this stage of every selection round relative quantities of phage moieties were determined by PCR product length analysis. Depending on the presence of either *tat* gene or *tet* spacer sequences, a PCR reaction with very short extension time using primers complementary to the *pel*B-, *tet*-spacer, and *gp3*-sequences, the resulting product is either 345 or 287 basepairs in length. From the 1:5000 as well as from the 1:50000 pAK-tat-wt:pAK-tat-mt5cys mixtures more than 90 % of the tested phages contained the *tat* gene after four rounds of selection and amplification. As additional control, a third mixture contained a 1:5000 dilution of pAK-wt-tat with pAK-tat-mt4arg, which displays a Tat variant with greatly

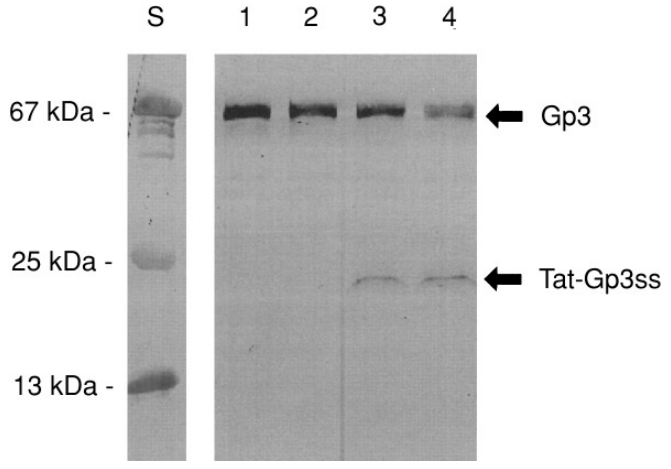

FIG. 2. Expression of Gp3ss-Tat and incorporation of the fusion protein into phage particles. Samples of about 10^{11} phage particles (VCS-M13, 1; pAK200, 2; pAK-tat-wt, 3; pAK-tat-mt5cys, 4) were separated by a denaturing 15% SDS-polyacrylamide gel electrophoresis, electrophoretically transferred to a nitrocellulose membrane, and immunostained with a monoclonal mouse anti-Gp3 antibody (MoBiTec, Göttingen, Germany) at a dilution of 1:1000. Gp3-anti-Gp3 antibody complexes were detected by treatment of the filter with rabbit anti-mouse IgG-alkaline phosphatase conjugate (DAKO, Glostrup, Denmark; 1:1000 dilution) and subsequent development with nitrotetrazolium blue and 5-bromo-4-chloro-indolylphosphate. Molecular weight standard proteins (S) were separated and blotted together with phage proteins but stained with Ponceau S (Merck, Darmstadt).

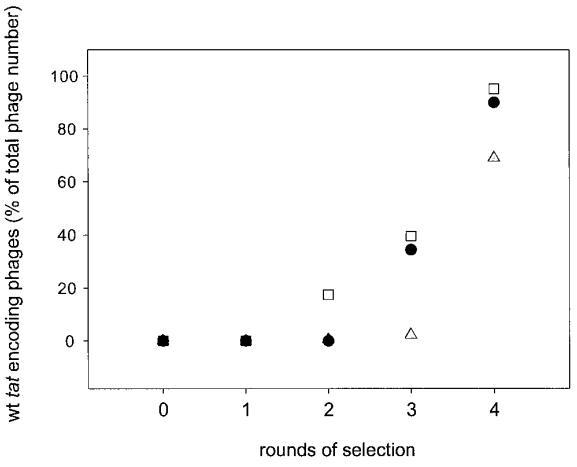

FIG. 3. Enrichment of HIV-1 Tat displaying phages from a 1:5000 (white rectangles) and 1:50000 (black circles) mixture with phages not displaying Tat and a 1:5000 mixture with phages displaying a Tat variant with greatly reduced affinity to TAR RNA (grey triangles). The percentage of phages carrying the wild type *tat* gene after each selection round was determined by either PCR product length analysis or restriction assay analysis. The results are shown before the selection procedure (round 0) and after each round of amplification and selection.

reduced affinity to TAR RNA due to exchange of arginines 53, 55, 56 and 57 to alanines, yielded enrichment of phages carrying wild type Tat to 70 % after 4 rounds of selection and amplification (Fig.3). Distinction between pAK-tat-mt4arg and pAK-tat-wt was easier and statistically more reliable using a restriction assay analysis because pAK-tat-mt4arg contained a single *Not*I restriction site which was not present in pAK-tat-wt. For the next round of the selection procedure, new phage particles were produced adding VCS-M13 helper phage.

Enrichment factors can be deduced from figure 3 to be in average between 25 and 30-fold for the first two and three rounds of the 1:5000 and 1:50000 pAK-tat-wt:pAK-tat-mt5cys selections, respectively. In the case of the 1:5000 pAK-tat-wt:pAK-tat-mt4arg selection, enrichment factors of only 5 could be obtained for the first three rounds suggesting that the Tat variant with residues 53, 55, 56 and 57 mutated from arginines to alanines has residual RNA binding activity. Although factors of 10^5 are reported (40), enrichment factors of 10 to 100 are much more common (41). Presence of very high excess (100-fold, by weight) of non-specific RNA during the selection procedure and the high affinity of HIV-1 Tat even to non-specific RNA certainly contributed to the comparably low enrichment factor of about 30, which still is enough to obtain more than a theoretical 10^8 fold enrichment after six rounds of selection and amplification.

The result shows three important points: First, Tat is displayed on the phage particles. Second, displayed Tat is functional. And third, using the pAK200 phagemid system (35) the high and odd number of cysteine residues of HIV-1 Tat does not interfere with correct phage display of the protein.

The present work serves as a basis for comprehensive investigations of the role of amino acid residues in HIV-1 Tat for affinity and specificity to TAR RNA. By randomizing certain residues or groups of residues at a time, several questions can be addressed. Are affinity and selectivity distinguishable? Are certain secondary structure types preferred in RNA recognition? These studies will further show, whether a Tat variant can be obtained that binds even tighter and more specifically to TAR, potentially leading to substances capable of successfully competing with HIV-1 Tat for binding to TAR. At the end of those investigations and similar ones with other model systems the knowledge of protein-RNA interactions may lead to a better understanding of the requirements for protein-RNA recognition.

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