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Structural requirements for conserved Arg^{52} residue for interaction of the human immunodeficiency virus type 1 trans-activation responsive element with trans-activator of transcription protein (49–57) Capillary electrophoresis mobility shift assay

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Abstract

A sensitive capillary electrophoresis mobility shift assay (CEMSA) for qualitative study of the interaction between the trans-activation response element (TAR) and the trans-activator of transcription protein (Tat) has been presented. The human immunodeficiency virus type 1 (HIV-1) Tat promotes elongation of viral mRNAs binding to the TAR. It has been suggested that a single, conserved arginine residue (presumably Arg^{52}) within the arginine-rich region (ARR) of Tat plays the major role for the Tat–TAR recognition. To study structural requirements of the Arg^{52} position, Tat(49–57)–NH_2 analogues substituted with nonencoded amino acids at the Arg^{52} position have been synthesized and their interaction with TAR has been studied by CEMSA. Using a linear polyacrylamide-coated capillary and a sieving polymer containing separation buffer, well separated and shaped peaks of free and bound TAR RNA were obtained. In the presence of Tat1 peptide bearing the native sequence of Tat(49–57) a significant shift of migration time of TAR from 18.66 min (RSD=1.4%) to 20.12 min (RSD=2.4%) was observed. We have found that almost every substitution within the guanidino group of the Arg^{52} [L-Arg^{52}→Cit, →Orn, →Arg(NO_2), →Arg(Me_2)] strongly disrupted or abolished the TAR–Tat peptide interaction. Enantiomeric substitution, L-Arg^{52}→D-Arg was the only one which notably promoted TAR–Tat peptide interaction. The results demonstrate that the specific net of hydrogen bonds created by the guanidinio group of conserved Arg^{52} plays a crucial role for TAR–Tat HIV-1 recognition. The newly developed procedure describes for the first time use of CE to monitor RNA–peptide complex formation. The methodology presented should be generally applicable to study RNA–peptide (protein) interaction.

Keywords: RNA–peptide interactions; Peptide–RNA interactions; Capillary electrophoresis mobility shift assay; Trans-activation responsive element; Arginine; Peptides; Proteins

1. Introduction

The early phase of the human immunodeficiency

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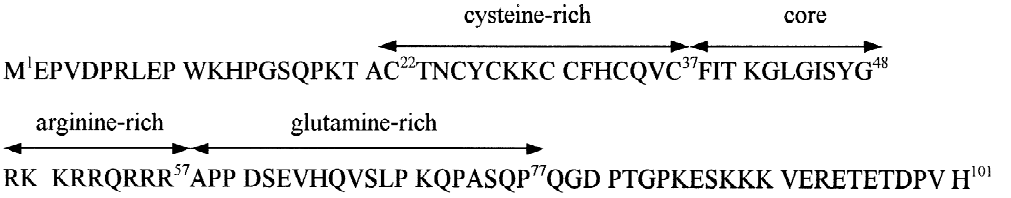
virus type 1 (HIV-1) replication cycle is controlled by the viral regulatory trans-activator of transcription protein (Tat) [1–8]. Tat is a potent viral trans-activator of 86–101 amino acids which binds to the trans-activation response element (TAR) RNA (Fig. 1) [9]. The basic role of Tat is to promote effective elongation of viral mRNA (vmRNA) during transcription of proviral DNA integrated with a human chromosome [1]. In vivo and in the absence of Tat, the transcription complex is non-processive and stops after passing 60–80 nucleotides of the viral long terminal repeats (LTR) sequences [10]. Tat comprises a few functional regions: a cysteine-rich region (22–37), a core region (38–48), an arginine-rich region (ARR) (49–57) and a glutamine-rich region (58–77) (Fig. 1) [2]. The ARR of Tat plays a dual role during the viral replication cycle. It contains a nuclear localization signal (NLS) which facilitates Tat's nuclear import and its proper nucleus/nucleous localization [11]. Mutations within the ARR reduce or abolish Tat trans-activation activity and appear to locate Tat primarily in the cytoplasm, not in the nucleus [12–15]. The ARR is also directly involved in binding of the trans-activation response element (TAR), a 59-base pair (bp) hairpin-bulge structure located at the 5′-end of all vmRNAs (Fig. 2) [16–19]. A 23-nucleotide TAR fragment (+18)–(+40) is sufficient to bind Tat and for in vivo trans-activation [20]. Tat recognizes TAR's trinucleotide bulge of sequence $U^{23}CU^{25}$ and induces a conformational change in the RNA during this interaction [16]. The invariant U^{23} position is critical for Tat binding and trans-activation in vitro, and cannot be substituted by any other nucleotide [21–23]. Binding of Tat to TAR is mediated by the highly basic ARR sequence: $R^{49}KKR^{52}RQRRR^{57}$ [6]. Scrambling and Ala/Lys-scanning studies of Tat ARR peptides suggest that a single, properly positioned arginine residue within the ARR (presumably Arg^{52}), provides the only sequence specific contact with the TAR bulge, although additional structural elements like the core region Tat(38–48) and the presence of cellular proteins are necessary for highly specific and processive TAR–Tat interaction [24–31]. Calnan et al. suggest that the single arginine residue within the ARR forms a specific network of hydrogen bonds called ''an arginine fork'' which is directly responsible for the specificity of Tat–TAR interaction [27]. CD and NMR studies of the TAR–Tat HIV-1 interaction demonstrated that simultaneous changes in the conformations of TAR and the Tat's ARR are the consequence of the recognition process [25,29,32]. Short Tat ARR peptides and even free argininamide or guanidine are able to induce conformational changes in TAR, similar to those observed during the TAR–wild-type Tat interaction [33].

The tertiary structures of free Tat, only free and bound TAR are relatively well known [32,34]. However, a detail structure of the TAR–Tat complex is still not yet available. NMR studies of the Tat HIV-1 protein revealed that the ARR adopted an unordered conformation and the Arg^{52} was exposed on an outer protein surface [34]. The structure of the ARR complexed with TAR is not known. It has been postulated that the ARR adopts an extended helical conformation in the TAR–Tat HIV-1 complex. There is still not enough structural and biochemical data to fully confirm this suggestion. Short ARR peptides displayed low ability to adopt helical conformation in aqueous solution, although the solved NMR structure of unbound hybrid peptide composed of the ARR Tat HIV-1 and the core regulatory domain of Tat EIAV showed that the Tat ARR fragment was able to adopt a stable helical conformation [33,35,36].

Capillary electrophoresis (CE) has become a powerful analytical technique in biochemical studies. Although CE has been previously used to study RNA biochemistry, only one example of RNA–protein interaction investigation has been reported [37]. Unfortunately, the authors did not observe RNA–protein complex directly but concluded about its formation monitoring changes in the peak of unbound RNA. Here, we report a CE mobility shift assay (CEMSA) study of TAR RNA interaction with Tat ARR peptides substituted at the Arg^{52} position. We focused on the Arg^{52} position to investigate its role as the driving force of TAR–Tat recognition. A change in TAR's peak electrophoretic mobility was used to monitor TAR–Tat peptide complex formation. The CEMSA observation of TAR–Tat recognition was extremely sensitive to substitutions at the Arg^{52} position and indicated that the Arg^{52} was the source of specificity within the ARR for Tat–TAR interaction.

(A)



(B)

Arg[49]-Lys-Lys-**Arg**[52]-Arg-Gln-Arg-Arg-Arg[57]-NH_2	Tat1
Arg[49]-Lys-Lys-**D-Arg**[52]-Arg-Gln-Arg-Arg-Arg[57]-NH_2	Tat2
Arg[49]-Lys-Lys-**Cit**[52]-Arg-Gln-Arg-Arg-Arg[57]-NH_2	Tat3
Arg[49]-Lys-Lys-**Orn**[52]-Arg-Gln-Arg-Arg-Arg[57]-NH_2	Tat4
Arg[49]-Lys-Lys-**Arg(Me)$_2$**[52]-Arg-Gln-Arg-Arg-Arg[57]-NH_2	Tat5
Arg[49]-Lys-Lys-**Har**[52]-Arg-Gln-Arg-Arg-Arg[57]-NH_2	Tat6
Arg[49]-Lys-Lys-**Arg(NO_2)**[52]-Arg-Gln-Arg-Arg-Arg[57]-NH_2	Tat7

(C)

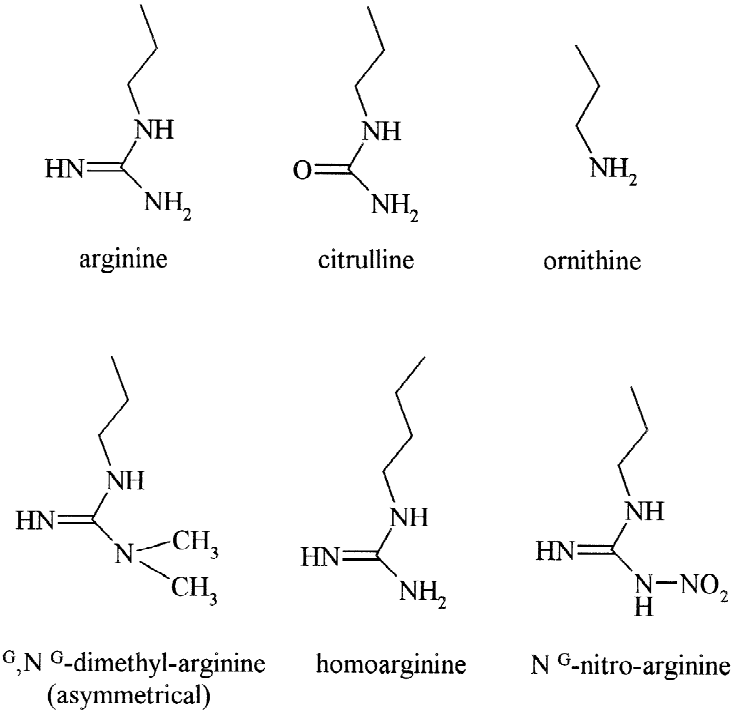


Fig. 1. Primary structure of Tat HIV-1 (A) (SF162 isolate), Tat-peptides (B) and the structure of unprotonated side chain of amino acid residues used to substitute the Arg[52] (C).

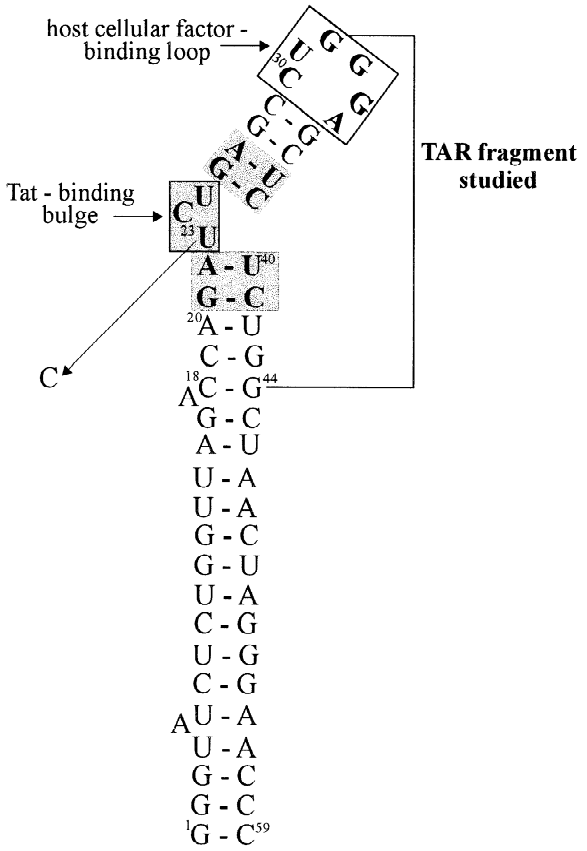


Fig. 2. Secondary structure of TAR RNA HIV-1. Highlighted nucleotides are essential for specific binding of Tat. Mutation U23→C of TAR_{CCU} used as a negative control for TAR–Tat interaction is shown.

2. Materials and methods

2.1. TAR and Tat peptide synthesis

A 27-nucleotide TAR RNA HIV-1 of sequence: 5′-CCAGAU^{23}CUGAGCCUGGGAGCUCUCUG-3′ containing the sequence of (+18)–(+44) of HIV-1 LTR and TAR mutant U^{23}→C (TAR_{CCU}) was purchased from Commonwealth Biotechnologies (USA). Tat ARR peptides with substitutions: L-Arg^{52}→D-Arg^{52}, →Cit, →Orn, →Arg(NO_2), →Arg(Me_2), →Har, Tat1 through Tat7 (Fig. 1), were synthesized as C-terminal amides by the solid-phase method using the 9-fluorenylmethoxycarbony (Fmoc)/TBTU protocol, as described previously [38].

2.2. TAR–Tat peptide complex preparation

To investigate concentration-dependent binding specificity, different concentrations (2.1–210 μ*M*) of Tat peptides were tested for TAR complex formation. A TAR–Tat peptide complex was formed by mixing pre-formed TAR (heated to 80 °C for 3 min and then cooled slowly to room temperature) with Tat peptide in binding buffer (10 m*M* Tris–HCl, 70 m*M* NaCl, 20 m*M* EDTA, 5% glycerol, pH 7.5). TAR–Tat peptide complex was incubated for 30 min at 0 °C before CE analysis. Final TAR concentration was 2.1 μ*M*. All solutions were prepared with Milli-Q water.

2.3. CEMSA of TAR–Tat peptide interaction

CEMSA experiments were carried out on a Beckman P/ACE 2100 capillary electrophoresis system using a BioCap 57 cm×75 μm linear polyacrylamide (LPA) coating capillary (Bio-Rad Labs., USA), 50 cm to the detector. The running buffer (267 m*M* Tris–borate with polymer modifier from Bio-Rad Labs.), with pH 8.3 was adjusted to pH 7.1 using solid boric acid. Reverse polarity, a constant voltage of 15 kV, a temperature of 20±0.1 °C and electrokinetic injection for 20 s at 10 kV were used. Free and bound TAR was detected at 254 nm. Before use, the capillary was pre-treated with water for 20 min, the washing buffer (the capillary wash solution from Bio-Rad Labs.) for 5 min, again with water for 5 min and finally with the running buffer for 5 min. The capillary was washed between runs with the running buffer for 3 min. All experiments were in triplicate. Relative standard deviation (RSD) was calculated from a series of three experiments carried out with the same sample in 1 day. Solutions were filtered through a 0.22-μm PTFE membrane prior to use.

3. Results

A set of Tat ARR peptide analogues substituted at the Arg^{52} position has been synthesized by the solid-phase method to determine structural requirements of the Arg^{52} for Tat–TAR HIV-1 recognition (Fig. 1). TAR–Tat peptide analogue interaction has been analyzed using CEMSA. To achieve good resolution

and shape of free and bound TAR peaks, LPA-coated capillary and sieving matrix containing the separation buffer have been applied. A single, well-shaped peak of TAR with migration time t_m=18.66 min (RSD=1.4%) was clearly visible at TAR sample concentration of 2.1 μ*M* (Fig. 3). With reverse polarity (anode at the detection side) under the experimental conditions applied, only TAR peak was visible. Free TAR migrates faster than its complexed form as would be expected given the smaller charge to mass ratio of the complex and the binding was observed as electrophoretic mobility shift of TAR's peak (Fig. 3). The peak of the complex did not change significantly between 30 min and 3 h, indicating that equilibrium had been reached within 30 min (data not shown). All samples were, therefore incubated for 30 min at 0 °C before CEMSA analysis.

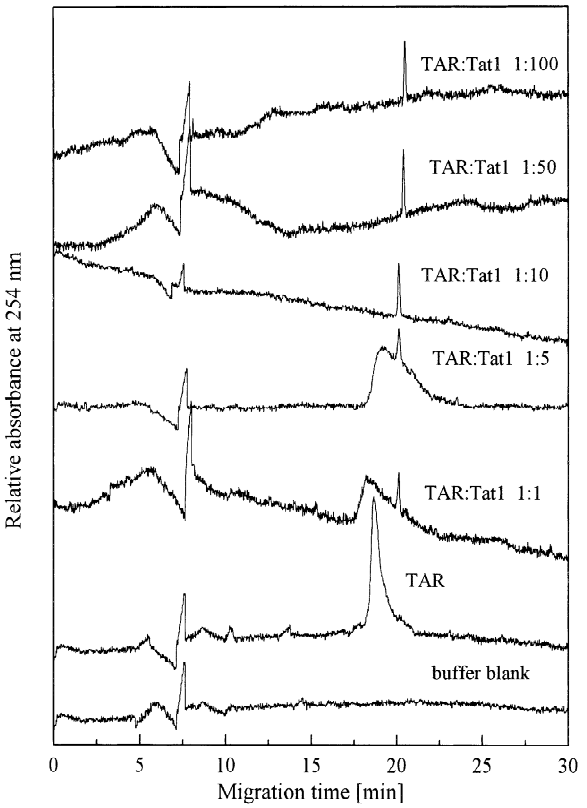


Fig. 3. Electropherogram of TAR–Tat1 peptide interaction. The conditions used were as follows: Beckman P/ACE 2100 capillary electrophoresis system, BioCap LPA coating capillary (Bio-Rad Labs.) 57 cm (50 cm to the detector)×75 μm, running buffer (267 m*M* Tris–borate with polymer modifier from Bio-Rad Labs.), pH 8.3 was adjusted to pH 7.1 using solid borate acid, reverse polarity, constant voltage 15 kV, temp. 20±0.1 °C, electrokinetic injection for 20 s at 10 kV, detection 254 nm. Molar ratio of TAR/Tat1 peptide sample analyzed is shown.

The effect of peptide concentration on the efficiency of TAR–Tat peptide complex formation was evaluated in the peptide concentration range of 2.1–210 μ*M*, an equivalent of 1- to 100-fold of the peptide excess over TAR. The peak of unbound TAR dominated at low Tat1 peptide excess (TAR/Tat1 molar ratio of 1:1 to 1:5). Increasing the Tat1 peptide concentration promoted the complex formation. At a TAR/Tat1 molar ratio 1:10, only a peak of the complex (t_m=20.12 min, RSD=2.4%) was observed. Incubation of TAR with different amounts of Tat1 peptide yields only the single peak of the complex even at high excess (100-fold) of the peptide, indicating that only specific interaction has occurred (Fig. 3). In the presence of urea (200 m*M*), the complex dissociated and only free TAR was observed (data not shown). Free peptide migrates in the opposite direction to RNA and is not observed under CEMSA conditions.

It has been shown previously that TAR–Tat recognition was TAR's bulge sequence specific [21–23]. TAR_{CCU} mutant (U^{23}→C) binds wild-type Tat non-specifically and has no affinity for Tat ARR peptides [18,25,26]. We observed that TAR_{CCU} did not interact with a low excess of Tat1 peptide (Fig. 4). However, at 100-fold Tat1 peptide excess, multiple broad peaks which may reflect weak, non-specific TAR_{CCU}–Tat1 interactions were observed.

Among all peptides studied, Tat2 bearing enantiomeric substitution L-Arg^{52}→D-Arg, was the only one which notably promoted TAR–Tat peptide interaction. TAR–Tat2 complex formation was clearly visible at equimolar concentrations of 2.1 μ*M* (Fig. 5). The peak of the TAR–Tat2 complex was more intense compared to the TAR–Tat1 peak recorded under the same conditions. At a fivefold excess of Tat2 peptide, the peak of the TAR–Tat2 complex dominates. At a TAR/Tat2 molar ratio above 1:10, all TAR was bound.

An extension of the aliphatic side chain of the Arg^{52} of Tat6 peptide, by introducing an additional methylene group (Arg^{52}→Har), only moderately decreased the peptide binding affinity for TAR (Fig.

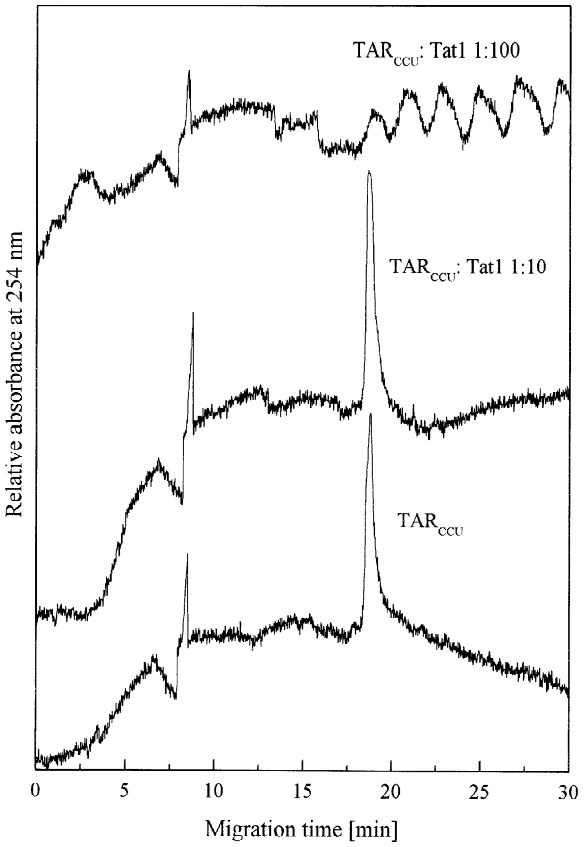


Fig. 4. Electropherogram of TAR_{CCU}–Tat1 peptide interaction. The conditions were the same as for Fig. 3. Molar ratio of TAR_{CCU}/Tat1 peptide sample analyzed is shown.

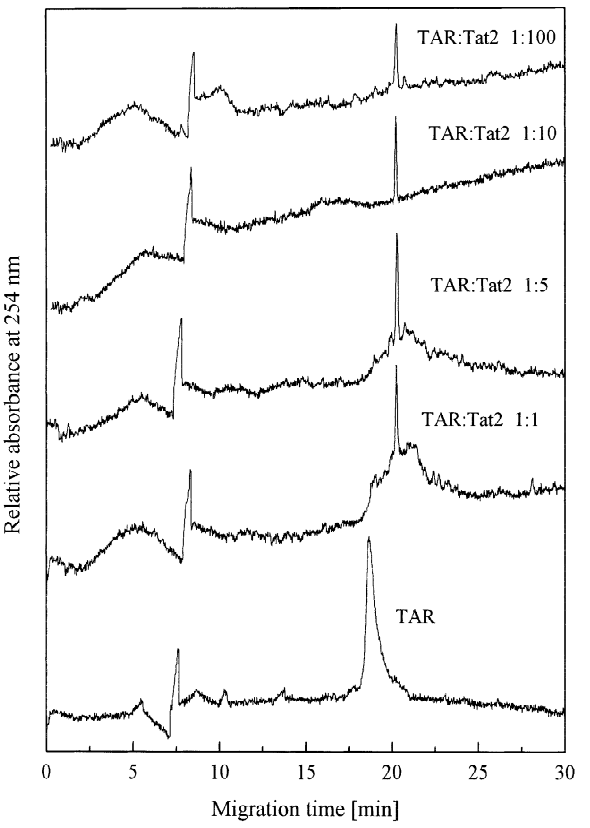


Fig. 5. Electropherogram of TAR–Tat2 peptide interaction. The conditions were the same as for Fig. 3. Molar ratio of TAR/Tat2 peptide sample analyzed is shown.

6). Effective TAR–Tat6 complex formation was observed at a 10-fold excess of the peptide. Under these conditions, equilibrium between unbound and complexed TAR was observed. At a TAR/Tat6 molar ratio above 1:50, only a single peak of the complex was observed.

Replacing the guanidino group of the Arg^{52} by the ureimido group of Cit^{52} ($Arg^{52}\rightarrow Cit$), strongly decreased the peptide affinity for TAR (Fig. 7). At a TAR/Tat3 molar ratio of 1:10, no trace of complex formation was detected. Further increase in Tat3 peptide concentration generated TAR's peak broadening and tailing. Only a weak sign of TAR–Tat3 complex formation has been observed as a small, weakly-shaped peak (t_m=20.25 min). At a 50-fold excess of Tat3 peptide, TAR–Tat3 complex was fully formed. Further increasing the Tat3 peptide concentration (TAR/Tat3 molar ratio 1:100) strongly decreased the intensity of the complex peak.

Substitutions, $Arg^{52}\rightarrow Orn$ which replaces the guanidino group of the Arg^{52} by the amine group of Orn^{52}, $Arg^{52}\rightarrow Arg(Me)_2$ where two methyl groups were asymmetrically introduced into the guanidino group and $Arg^{52}\rightarrow Arg(NO_2)$ where a nitro group was introduced into the guanidino group of the Arg^{52}, abolished Tat-peptide binding to TAR (Fig. 8). Only at high peptide excess, a broadening/tailing of TAR peak or multiple peaks interpreted as nonspecific interactions were observed. Over a TAR–Tat peptide molar ratio of 1:100, insoluble aggregates (sample turbidity) were visible and no peaks were observed by CEMSA after sample centrifugation (data not shown).

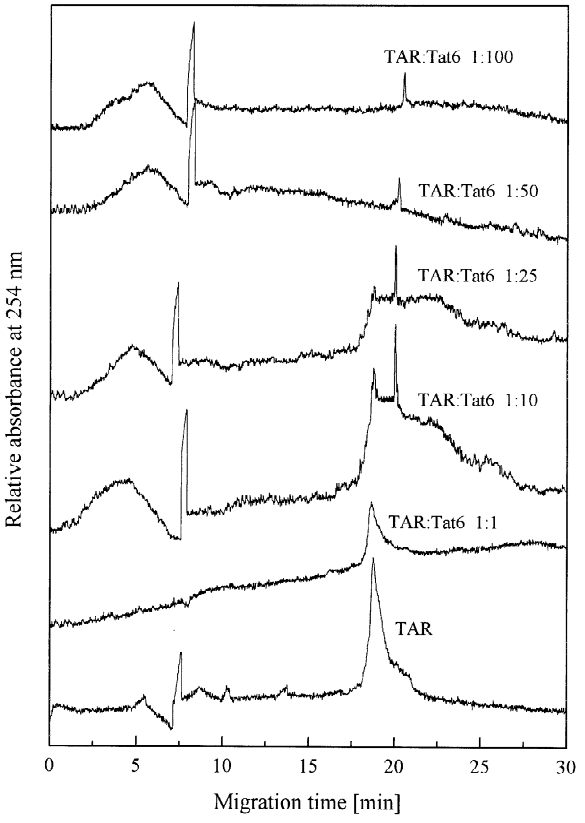


Fig. 6. Electropherogram of TAR–Tat6 peptide interaction. The conditions were the same as for Fig. 3. Molar ratio of TAR/Tat6 peptide sample analyzed is shown.

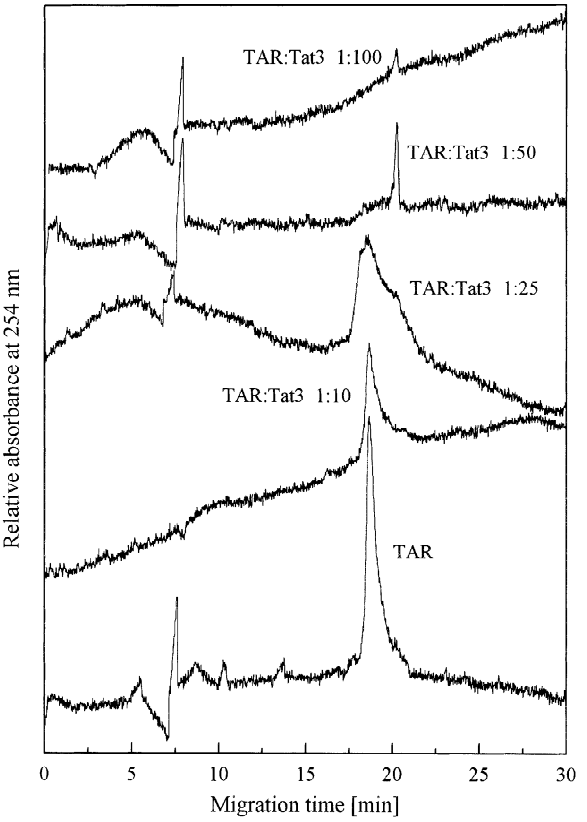


Fig. 7. Electropherogram of TAR–Tat3 peptide interaction. The conditions were the same as for Fig. 3. Molar ratio of TAR/Tat3 peptide sample analyzed is shown.

4. Discussion

The effective elongation of HIV-1 mRNAs transcripts is controlled by Tat–TAR interaction. The interaction is mediated through specific binding of the 5′-end vmRNA element TAR and the arginine-rich region (ARR) of Tat. In this report we have shown that the Arg^{52} of Tat HIV-1 is the source of specificity for the ARR–TAR interaction. All Tat peptides substituted at the Arg^{52} position (except Tat2 with D-Arg^{52} substitution) had decreased affinity for TAR. This observation supports a model where a precisely positioned guanidino group of the Arg^{52} is almost entirely responsible for the specificity of TAR–Tat interaction, although some experiments suggested contribution of the core region of Tat.

CEMSA is shown to provide a quick, sensitive and precise method to study RNA–peptide interaction. The use of LPA-coated capillary and sieving matrix-containing separation buffer resulted in sharp peaks of free and bound TAR. This was true only when a specific and strong interaction of TAR–Tat peptide took place. All substitutions of the Arg^{52} (except L-Arg→D-Arg) strongly affected Tat peptide affinity for TAR. Interestingly, Tat1 and Tat2 peptides had a comparable affinity for TAR. These results strongly suggest that inherent flexibility of the ARR allows the Arg^{52} residues of enantiomeric configuration to fit precisely into the bulge. It is worth noting that Tat6 peptide with Arg^{52}→Har substitution bearing additional methylene group in the side chain of homoarginine compare to arginine, reflects only moderate binding affinity decrease and

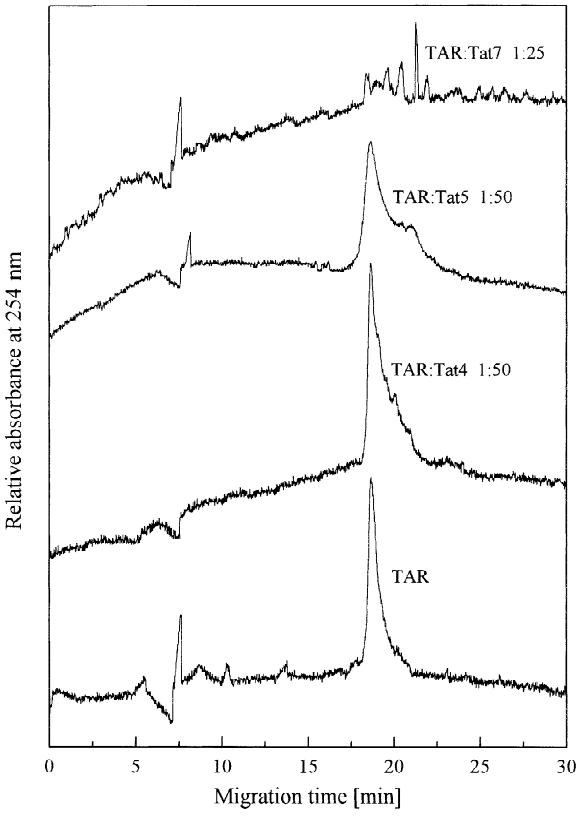


Fig. 8. Electropherogram of TAR–Tat4, 5, 7 peptides interaction. The conditions were the same as for Fig. 3. Molar ratio of TAR/Tat peptide samples analyzed is shown.

retains its specificity for TAR. This observation shows that there is enough space in the arginine binding pocket of TAR to accommodate an additional methylene group of the side chain of amino acid residue at the position 52, although a newly formed net of hydrogen bonds is probably less effective than the wild-type one proposed in the ''arginine fork'' model. Substitutions Arg^{52}→Cit, →Orn, →$Arg(Me)_2$, →$Arg(NO_2)$ strongly or completely abolished TAR binding. Thus, it is likely that precisely locating the net of hydrogen bonds between the Arg^{52} and the bulge of TAR is the major determinant for Tat–TAR recognition. Based on the results obtained, we concluded that the guanidino group of the Arg^{52} was the main source of specificity of the TAR–Tat interaction. Stereochemical tolerance for the Arg^{52} position probably arose from high flexibility and unordered conformation of Tat's ARR.

The Tat1–TAR complex was stable under CEMSA conditions. The half-life of a similar complex, TAR–Tat(48–61), determined by previous SGE experiments was only 1.4 min [25,39]. This shows that the complex of TAR with short ARR peptides is relatively unstable under SGE experimental conditions. Using CEMSA, a high precision of TAR–Tat complex characterization was obtained. Although peak area repeatability was not studied, a high precision of migration time of free and complexed TAR was obtained, with RSD of 1.4 and 2.4%, respectively. We observed that Tat1 peptide discrimination against wild-type TAR and its mutant TAR_{CCU} was higher than observed previously [39]. Tat(48–61) discriminated TAR_{CCU} mutant from the wild-type TAR with moderate (7- to 20-fold lower) specificity in SGE analysis [26,39]. A high discrimination level of TAR mutants by Tat1 peptide was observed during CE analysis. At TAR/Tat1 molar ratio 1:10, the complex was formed efficiently and no traces of such interaction were visible in the case of TAR_{CCU}/Tat1 under the same conditions. It is unclear whether the reason for the higher specificity was using thin LPA-coated capillary or other CEMSA conditions (separation buffer or voltage). These features define CE as a more accurate technique to study small RNA–peptide complexes than SGE. CEMSA results show that small perturbations of the guanidino group structure of the Arg^{52} cause dramatic changes in the peptide binding affinity. Even small substitutions like methylation (Arg^{52}→$Arg(Me)_2$) which preserve the overall positive charge of the Arg^{52}, strongly decrease TAR–Tat peptide binding. A negative effect of the arginine methylation presumably arose from removing two of five potential hydrogen bonds created by the guanidino group. One could conclude that overall positive charge of Tat's ARR was important but insufficient for TAR binding. This conclusion was supported by the observation that less basic Tat4 peptide (Arg^{52}→Cit) displayed strongly reduced but visible binding affinity for TAR. The ureimido group of Cit^{52} retains some of the hydrogen bonding potential of the guanidino group but lacks the positive charge. This implies that the specific role of the Arg^{52} is non exchangeable and the net of

hydrogen bonds created by the Arg^{52} is critical for TAR recognition.

Full-length Tat protein binds TAR with high affinity of dissociation constant in the nanomolar range [17]. Because of the difficulties in obtaining correctly folded Tat and its strong tendency to oxidize most TAR–Tat studies have focused on the use of Tat peptides containing the basic ARR sequence. Tat peptides bind TAR with lower affinity compared to native Tat, probably because other fragments of Tat like the core and glutamine-rich regions are involved in TAR binding [2,32]. Although one agrees that Tat peptides have lower affinities for TAR compared to native Tat, there is some confusion about the binding constant range of such an interaction. Reported K_d values are from nano- to micromolar range [17,26,32]. Although in this paper, we presented only qualitative data, the conditions of our CEMSA experiments suggested that TAR–Tat peptide interaction was of moderate, micromolar range.

5. Conclusion

CEMSA results revealed that TAR–Tat ARR peptide interactions were specific and there was only one arginine binding pocket in the TAR's bulge, suitable for Tat peptide binding. The approach presented to understand the nature of the Arg^{52} guanidino group interaction with TAR structure may be applied to design new, Arg-based compounds to modulate TAR–Tat interaction. Small peptides and peptidomimetics containing nonencoded amino acids are promising tools for artificial regulators of cellular processes involving RNA–protein interaction [40,41]. CEMSA provided a significant improvement in the speed and precision of RNA–peptide interaction analysis compared to slab gel electrophoresis. Although only qualitative data results are presented, the methodology should be applicable to obtain quantitative data on RNA–peptide (protein) interaction.

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