INTERACTION OF HIV TAT PEPTIDES WITH tRNA^{Phe} FROM YEAST

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We present data on the interaction of arginine-rich peptides of human immunodeficiency virus (HIV-Tat) with tRNA^{Phe} of *Saccharomyces cerevisiae*. We have found that tRNA forms complexes with the Tat1 peptide of amino acid sequence GRKKRRQRRRA and its mutants where R is replaced by D-arginine, citrulline or ornithine. The structure of tRNA–Tat1 complex was probed by specific RNases digestions and Pb^{2+}-induced cleavage of phosphodiester bond of guanosine. The nucleotide sequence UGGG located in the dihydrouridine loop of tRNA^{Phe} binds to Tat peptide and therefore is specifically protected against RNases and is not hydrolyzed by Pb^{2+} ion. It seems that the peptide–RNA complex formation depends on direct recognition of guanine moieties of tRNA with arginine residues. These interactions are similar to those observed in many DNA–protein complexes, but are different from those previously observed for TAR RNA–Tat complexes.

Key words: Tat peptides; TAR RNA; tRNA^{Phe}; Protein–nucleic acids interactions; HIV.

During last several years, the mechanism of protein–nucleic acids interactions was the subject of numerous studies. On the basis of crystallographic data of many complexes, several protein structural motifs responsible for specific recognition of DNA have been identified[1]. For DNA–protein complexes a recurring feature is a recognitory α-helix, which perfectly fits and binds into the major groove of B-DNA where amino acid side chains form specific hydrogen bonds and non-specific contacts with bases and backbone phosphates, respectively. In some cases, a high specificity of binding of DNA to protein has been achieved through bound water molecules[2]. Detailed analysis of crystal structures of various DNA–protein complexes was a basis to propose a code of recognition of transcription factors with DNA (refs[3–5]). It has been observed that the guanine

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and adenine residues form specific contacts with arginine and glutamine, respectively. A summary of recent knowledge of RNA–protein complexes can be found in literature[6].

Recent advances in understanding specific interactions in these complexes originate mostly from studies of three-dimensional structures of the complexes of glutaminyl-, aspartyl- and seryl-tRNA synthetases with their cognate tRNAs (refs[7–9]), R17 coat protein with 19-nucleotide RNA hairpin[10] and U1A protein domain with 20-nucleotide RNA hairpin[11]. A general conclusion from those data indicates that the tertiary structure rather than the nucleotide sequence of RNA is a critical element for specific RNA recognition by a cognate protein. Also several protein–RNA binding motifs for specific interaction with ribonucleic acid have been identified. One known domain consists of a short string of basic amino acids, mainly arginine residues[6,12] (arginine-rich motif, ARM). Similar motifs have been found in many other proteins as bacterial antiterminators, ribosomal proteins, coat proteins of RNA viruses, a human Tat (HIV-Tat) and a bovine Tat (BIV-Tat) immunodeficiency virus, and Rev (HIV-Rev) proteins[13,14]. The Tat protein activates expression of the HIV-1 genome by its specific binding to transactivation response RNA (TAR RNA) sequence, located at the 5′-end of the untranslated leader region of HIV mRNA (ref.[15]). The latter acquires a specific secondary structure involving a helical stem interrupted by a trinucleotide bulge terminated with the six-residue loop[15].

Some years ago the tertiary structure of TAR RNA in complex with arginine amide has been solved by NMR spectroscopy[16]. Hydrogen bonds between arginine and the guanine residues in position 26 and two phosphates (positions 22 and 23) have been identified. Binding of the amino acid induces structural changes in RNA and formation of the U23-A27-U38 base triple[16,17]. These data have not been confirmed in other studies of the HIV RNA-Tat (ref.[18]). On the other hand, computer modelling studies of the TAR RNA–Tat complex have demonstrated that it is possible to accommodate the α-helix of peptide into the RNA major groove, widened significantly by the bulge[19].

In order to get better insight into the RNA domain which binds to arginine-rich peptides, we used yeast $tRNA^{Phe}$. This model molecule not only contains (5′)-UGGG sequence in the dihydrouridine loop identical to that occurring in the TAR RNA molecule, but what is more important the tertiary structure of this molecule is solved and confirmed by various crystallographic studies. In addition, the oligonucleotide UGGG is involved in formation of the three-dimensional structure of yeast $tRNA^{Phe}$ (ref.[20]).

In our studies, we used synthetic peptides with amino acid sequence corresponding to the RNA-binding domain of HIV-1 Tat. We found that $tRNA^{Phe}$ forms complexes with these peptides. As shown by digestion of the complexes with specific RNases, the Tat peptides bind to dihydrouridine loop. These interactions resemble very much the guanosine–arginine recognition mode observed previously in numerous DNA–protein complexes, but are different from those proposed earlier for TAR-Tat, where protein

recognizes distorted helical RNA fragment. The binding of Tat peptides to $tRNA^{Phe}$ requires disruption of its 3D structure.

EXPERIMENTAL

Synthesis of Polypeptides

Tat1–4 peptides, having the amino acid sequences shown in Table I and Fig. 1 were synthesized manually on a cross-linked polystyrene resin (capacity 0.68 mmol/g) by the solid-phase method, using the Boc chemistry. The following side-chain protection groups were applied: benzyl (Ser, Thr), (2-chlorobenzyl)oxycarbonyl (Lys), cyclohexyl (Asp), tosyl (Arg) and *N*-π-benzylohexymethyl[21] (His). Synthesized peptides were cleaved from the resin by the HF procedure and lyophilized. The

TABLE I
Sequences of Tat model peptides

HIV-1 Tat	…^{39}RKLGLGISY**GRKKRRQRRRA**PQDSQTHQASLSKQPASQ^{76}…
Tat1 peptide	^{1}**GRKKRRQRRRA**^{11}

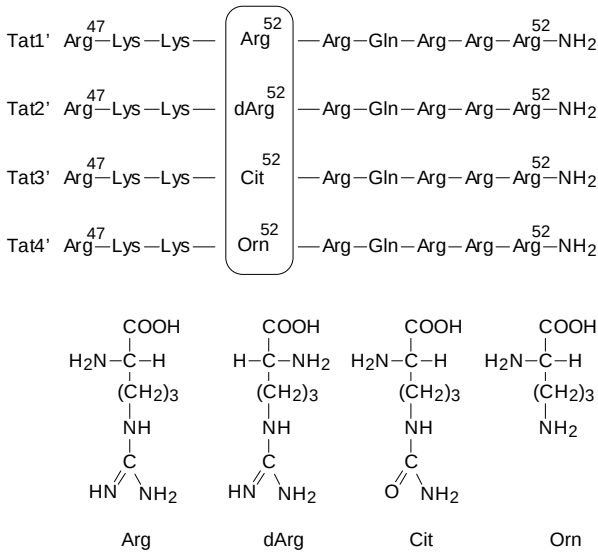

FIG. 1
Amino acid sequences of 9-amino acid long peptide Tat1′ and its mutants used in the experiments. Arginine corresponding to Arg^{52} in native HIV Tat protein was replaced by D-arginine, citrulline and ornithine in Tat2′, Tat3′ and Tat4′, respectively

crude product was desalted on a Sephadex G-25 column (2.8 × 105 cm) in 30% AcOH and chromatographed twice on preparative RP-HPLC using Vydac C-18 column (32 × 240 mm, 15–20 μm particle size). Separation was done in linear gradient of 0–15% acetonitrile in 0.1% TFA. Retention times were 8.8 and 5.4 min for RP-HPLC and capillary electrophoresis, respectively.

Molecular ions of the peptides were determined by fast atom bombardment mass spectrometry method (FAB MS) on an AMD-604 mass spectrometer. Calculated molecular ion weight for $C_{58}H_{114}N_{32}O_{13} + H^+$ was 1 468.8, experimentally determined value was 1 468.5.

Amino acid analysis of Tat1 was performed on a Beckman model 121M instrument and the following result was obtained: Glu1.10(1), Gly1.05(1), Ala1.00(1), Lys1.78(2), Arg5.85(6). The peptide was hydrolyzed with hydrochloric acid containing 1% of phenol at 110 °C for 24 h.

Isolation of RNA

The $tRNA^{Phe}$ extracted from yeast was purified on 15% polyacrylamide gel with 7 M urea in 0.05 M Tris-borate buffer. The sample of $tRNA^{Phe}$ was 3′-end labelled with [^{32}P]pCp and RNA ligase[22]. [^{32}P]-Labelled tRNA was purified by electrophoresis on 10% polyacrylamide gel (PAGE) with 7 M urea, eluted from the gel and renatured[23].

RNA Binding Reactions and Electrophoretic Mobility Gel Shift Assays

The $tRNA^{Phe}$–Tat peptide complex formation assay was performed at 22 °C for 40 min in pH 7.5 buffer (0.05 M Tris-HCl, 0.07 M NaCl, 0.001 M EDTA, 0.1% Nonidet P-40, $1 . 10^{-9}$ M $tRNA^{Phe}$, 2 μg of crude tRNA) with $3 . 10^{-9}$ M Tat peptide in total volume 10 μl. Analysis of the complexes was carried out on 0.7% agarose gel in 0.05 M Tris-borate buffer.

RNase Footprint Assay

In a footprint reaction of the tRNA–peptide complex, the following amounts of RNases were used: T1 ($2 . 10^{-4}$ U), V1 ($6 . 10^{-2}$ U) and S1 (3U) (Pharmacia). For localization of the Tat binding site, 4 μg of tRNA and 40 000 cpm of labelled $tRNA^{Phe}$, respectively, were digested with 0.04 U of T1 RNase in 20 mM sodium acetate buffer (pH 4.5, 7 M urea, 1 mM EDTA and 0.05% xylene cyanol) and analyzed on 10% polyacrylamide gel with 7 M urea in 0.09 M Tris-borate buffer.

Metal Ion-Induced RNA Hydrolysis

Labelled $tRNA^{Phe}$ was supplemented with 8 μg of non-specific RNA. Tat1 peptide was used at 0.1 mM, 0.5 mM or 0.8 mM final concentration. The complex formation reaction was performed as described above for 40 min, 1 μl of 5 mM $Pb(CH_3COO)_2$ was added for RNA hydrolysis. Reaction (15 min) was stopped by mixing with 10 μl of 8 M urea/dyes 20 mM EDTA and loaded on 10% polyacrylamide gel.

RESULTS AND DISCUSSION

Many recent studies are focused on understanding of the mechanism of protein–nucleic acids recognition. The most important issue here is to select proper model systems where detailed interactions can be identified and analyzed. The NMR data of the HIV TAR–Tat complex have suggested that the arginine residue of Tat recognizes the UCU bulge of RNA (ref.[17]). These interactions are totally different from those observed in

some DNA–protein complexes, where direct binding of guanine and arginine occurs[24]. To solve an intriguing question why interactions within RNA–protein and DNA–protein complexes are different and the arginine residues do not interact with the guanine-rich RNA loop, we turned to another RNA molecule for which many structural data are available including its three-dimensional structure[20], to the native $tRNA^{Phe}$ from yeast. It contains the (5′)UGGG nucleotides in the dihydrouridine loop. An identical string was found in the TAR RNA hairpin tip (Fig. 2).

For analysis of specificity of tRNA binding capacity, we prepared arginine-rich synthetic peptides Tat1 and Tat1′ containing 11 and 9 amino acid residues, respectively. In addition, Tat analogue mutants Tat2′, Tat3′ and Tat4′ substituted at position Arg52 with D-arginine, citrulline and ornithine, respectively, have been synthesized (Table I, Fig. 1). The amino acid sequence of these peptides is identical to the RNA binding motif of the HIV-1 Tat protein[25].

In order to check whether these peptides form complexes with $tRNA^{Phe}$, we carried out agarose gel shift assay. It shows that Tat1′ forms very stable complex with $tRNA^{Phe}$ at molar ratio 1 : 3 (Fig. 3, lanes 3–5). Tat2′ (D-Arg) binds to yeast $tRNA^{Phe}$ with smaller affinity than Tat1′ at molar ratio 1 : 5 (lanes 9–10). Higher excess of both Tat1′ and Tat2′ peptides (lanes 1, 2, 8) causes formation of aggregates. Tat2′ at concentration of 16.4 and 21.9 nM in reaction mixture (lanes 6, 7) precipitates $tRNA^{Phe}$. Tat3′ and Tat4′ form complexes with tRNA only with higher excess of peptide (23 : 1) (Fig. 3, lanes 11, 16).

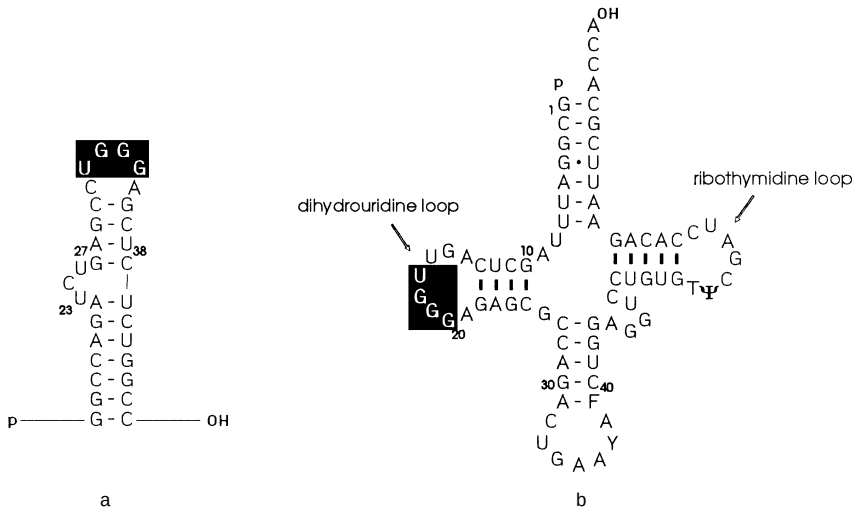

FIG. 2
The secondary structure of a TAR RNA of HIV-1 (ref.[17]), b $tRNA^{Phe}$ of yeast[20]. UGGG sequence is highlighted by black box

To determine the peptide binding site on $tRNA^{Phe}$, a limited specific RNase hydrolysis was used. We applied RNase T1 (guanosine specific), S1 (single-stranded RNA specific) and V1 (double-stranded RNA specific). In these experiments, we used Tat1 (11-aa) which formed more stable complexes than Tat1′ (9-aa) (Fig. 4). T1 RNase digestion of $tRNA^{Phe}$ shows two weak bands corresponding to nucleotides G18G19 (Fig. 4).

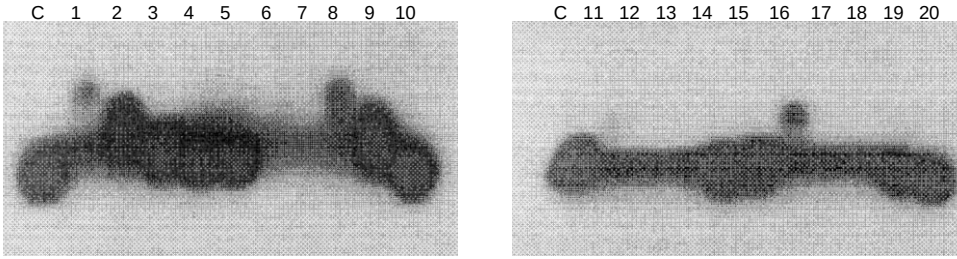

FIG. 3
Analysis of the binding of the Tat peptides to [3′-^{32}P]$tRNA^{Phe}$ on 0.7% agarose gel; Tat1′ lanes 1–5, Tat2′ lanes 6–10, Tat3′ lanes 11–15, Tat4′ lanes 16–20. Concentration of the peptide used in the experiments was as follows. Lanes 1, 6, 10, 16: 21.9 nM; 2, 7, 12, 17: 16.4 nM; 3, 8, 13, 18: 10.9 nM; 4, 9, 14, 19: 5.4 nM; 5, 10, 15, 20: 2.7 nM

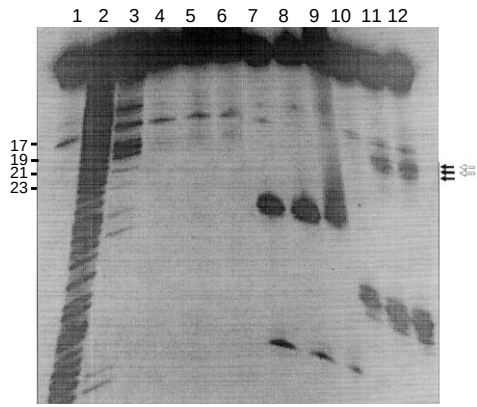

FIG. 4
Autoradiogram of 10% polyacrylamide gel with 7 M urea showing hydrolysis products of [3′-^{32}P]$tRNA^{Phe}$–Tat1 complex obtained by RNase T1 (lanes 4–6), RNase V1 (lanes 7–9) and RNase S1 (lanes 10–12). Lanes: 1 control $tRNA^{Phe}$ incubated in the reaction buffer (10 min/22 °C); 2 ladder; 3 limited hydrolysis of $tRNA^{Phe}$ with RNase T1; 4, 7, 10 $tRNA^{Phe}$; 5, 8, 11 $tRNA^{Phe}$ + 1.45 nM Tat1 peptide; 6, 9, 12 $tRNA^{Phe}$ + 3 nM Tat1 peptide. Differences in hydrolysis pattern of free tRNA and in complex are marked by arrows on the right site (open arrows indicate the nucleotides protected from hydrolysis with RNase T1 and full arrows the sites of enhanced digestion by RNase S1); the numbers on the left side correspond to nucleotides in primary sequence of $tRNA^{Phe}$ (see Fig 5)

They are not present in the digestion pattern of the complex. At the same time, there are no differences in the RNase V1 hydrolysis of tRNA free and in the complex. Interestingly, RNase S1, in contrast to T1 RNase, hydrolyzed $tRNA^{Phe}$ in complex with Tat1 at G17G18G19, but not free tRNA (Fig. 5). These results of specific cleavages of the complex with T1, S1, and V1 RNases clearly show that indeed the Tat1 polypeptide interacts directly with nucleotides occurring in dihydrouridine loop (D) of $tRNA^{Phe}$ molecule.

To go further in elucidation of the mechanism of the interaction of Tat peptide with D loop of tRNA, which is involved in a tertiary structure formation, we have carried out lead-induced cleavage reaction. The reaction can take place only when native structure of the molecule is preserved (Fig. 6). The location of cleavage sites was identified with RNase T1 ladder (lane T1). The Pb^{2+}-induced hydrolysis bands occur between nucleotides 17–16 and 16–15 of free $tRNA^{Phe}$ (lane 1), but after complex formation with Tat1 peptide (lanes 2–3) there are not cleavages at all.

Lack of the lead-induced cleavages we interpret as a result of disturbing the tertiary structure of tRNA by Tat peptide. From the crystallographic structure of yeast $tRNA^{Phe}$, it is known that G18 and G19 of the loop D and C56 and Ψ55 in the ribothymidine loop (T) form hydrogen bonds, respectively[20]. If so, lack of cleavage at G18 and G19 by RNase S1 in the absence of Tat1 and a strong hydrolysis in the presence of the polypeptide suggest conformational changes of tRNA leading to accessibility of the sugar–phosphate backbone in the complex, where the guanosine residues are protected against T1 RNase. From our previous CD spectra analysis of Tat1–$tRNA^{Phe}$ complex, we know that some differences in the tertiary structure of tRNA free and bound to the peptide exist[26]. Therefore it is not suprising that the nucleotides in loop D are not hydrolyzed

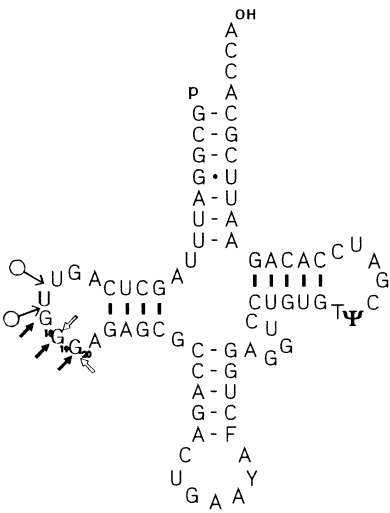

FIG. 5

RNase and Pb^{2+}-induced hydrolysis data of the $tRNA^{Phe}$ from yeast superimposed on the secondary structure of the molecule. Open arrows indicate the nucleotides protected from hydrolysis with RNase T1 and full arrows the sites of enhanced digestion by RNase S1. Ring arrows mark the positions hydrolyzed by Pb^{2+} in free molecules of $tRNA^{Phe}$

by Pb^{2+} ions, which is known as a very sensitive probe for the correct folding of tRNA. A detailed mechanism of Pb-cleavages has been proposed, in which $Pb(OH)^{+}$ coordinated to U59 and C60 abstracts a proton from the 2′-OH group of D17 to facilitate phosphodiester hydrolysis *via* a cyclic phosphodiester intermediate[27,28]. In such case, lack of lead-induced hydrolysis of $tRNA^{Phe}$ in presence of the Tat peptide strongly suggests that the distance between the dihydrouridine loop (D) and ribothymidine loop (T) in the complex has to be longer than in free tRNA. The tertiary hydrogen bonds of

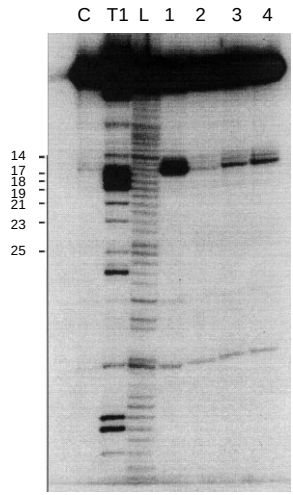

FIG. 6
Autoradiogram of 10% polyacrylamide gel with 7 M urea showing Pb^{2+}-induced hydrolysis products of [3′-^{32}P]$tRNA^{Phe}$–Tat1 complex. Lanes: C control $tRNA^{Phe}$ in water; T1 limited hydrolysis of $tRNA^{Phe}$ with RNase T1; L alkali ladder; 1–4 hydrolysis of $tRNA^{Phe}$ free (lane 1) and in presence of Tat1 peptide (lanes 3–4) induced by Pb^{2+}. The conditions of the reactions are as described in Experimental. The numbers on the left side correspond to nucleotides in primary sequence of $tRNA^{Phe}$ (see Fig. 5)

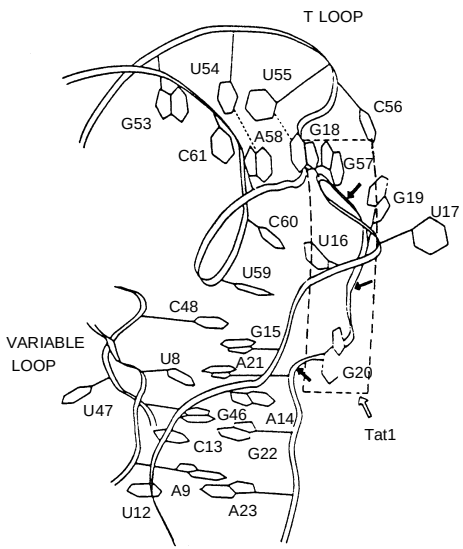

FIG. 7
Three-dimensional structure of loops D and T of $tRNA^{Phe}$ (ref.[20]). According to the proposed mode of interaction of Tat1 peptide with $tRNA^{Phe}$, the peptide is situated in the cleft between the loops D and T. Arrows denote sites accessible for RNase S1 in complex

$tRNA^{Phe}$, G19-C56 and G18-Ψ55 between D and T loops are disrupted in the complex of $tRNA^{Phe}$ with Tat peptide. Taking this into account, as well as the results of specific RNase hydrolysis, we propose that the peptide is located in the cleft between loops D and T (Fig. 7).

We suppose that the Tat peptides form hydrogen bonds with $tRNA^{Phe}$ in a similar way to the Zif268 protein in complex with DNA. Most interactions take place between guanosine and arginine residues[24].

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