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Molecular recognition of amino acids by RNA aptamers: The evolution into an L-tyrosine binder of a dopamine-binding RNA motif

CECILIA MANNIRONI, CHIARA SCERCH, PAOLO FRUSCOLONI, and GLAUCO P. TOCCHINI-VALENTINI

Istituto di Biologia Cellulare, Consiglio Nazionale delle Ricerche, "Adriano Buzzati-Traverso" Campus, I-00016 Monterotondo Scalo, Rome, Italy

ABSTRACT

We report the evolution of an RNA aptamer to change its binding specificity. RNA aptamers that bind the free amino acid tyrosine were in vitro selected from a degenerate pool derived from a previously selected dopamine aptamer. Three independent sequences bind tyrosine in solution, the winner of the selection binding with a dissociation constant of 35 μM. Competitive affinity chromatography with tyrosine-related ligands indicated that the selected aptamers are highly L-stereo selective and also recognize L-tryptophan and L-dopa with similar affinity. The binding site was localized by sequence comparison, analysis of minimal boundaries, and structural probing upon ligand binding. Tyrosine-binding sites are characterized by the presence of both tyrosine (UAU and UAC) and termination (UAG and UAA) triplets.

Keywords: affinity chromatography; amino acid; RNA aptamers; selection-amplification

INTRODUCTION

The ability of RNA to form specific binding sites for free amino acids is now well established and RNA sites for various amino acids have been recently isolated from random RNA pools by selection-amplification techniques (for a review, see Yarus, 1998).

To date, the best characterized RNA site for a free amino acid is the arginine-binding site. Eight independent arginine RNA aptamers were isolated by in vitro selection (Connell et al., 1993; Connell & Yarus, 1994; Famulok, 1994; Geiger et al., 1996; Tao & Frankel, 1996), which indicates that there are several different motifs that can generate specific RNA sites for arginine. Moreover, natural examples of arginine RNA sites are the highly conserved group I intron's guanosine cosubstrate sites, where the arginine site lies inside the guanosine site (Yarus, 1988; Hicke et al., 1989).

The capability of RNA to form amino acid-binding pockets, not only for the intensively polar side chain of arginine but also for aliphatic hydrophobic side chains, has been shown by the isolation of valine and isoleucine RNA aptamers (Majerfeld & Yarus, 1994, 1998). The well-characterized isoleucine-binding sites are highly specific, distinguish side chains of different shapes and polarity, and show affinities for the cognate amino acid not dissimilar to those of many proteins that bind hydrophobic ligands. In spite of the varied specificities featured by the RNA aptamers, conferred by definite functional groups, the overall structure of the described amino acid-binding sites is quite simple, and asymmetric internal loops are their most prominent structures.

We now report the isolation of RNAs able to bind the amino acid L-tyrosine. The tyrosine aptamers were obtained by in vitro evolution of a previously selected dopamine aptamer (Mannironi et al., 1997).

RESULTS

Selection of tyrosine aptamers

The starting RNA pool for L-tyrosine selection was derived from the partial randomization of the dopamine aptamer dopa2/c.1 (Mannironi et al., 1997), mutagenized at a level of 30%. The RNA random pool was selected on an L-tyrosine affinity matrix. Following extensive washes to remove unbound or weakly bound molecules, specifically bound RNAs were eluted in 1 mM

Reprint requests to: Glauco P. Tocchini-Valentini, Istituto di Biologia Cellulare, CNR, Campus "A. Buzzati-Traverso", Via E. Ramarini, 32, 00016 Monterotondo Scalo (Roma), Italy; e-mail: gtocchini@ibc.rm.cnr.it.

L-tyrosine. After 10 cycles of selection-amplification, a distinct peak of tyrosine elution was observed. After 13 cycles, 14% of the applied RNA was affinity eluted from the column. No further enrichment was obtained after two more cycles of selection. RNA from the 13th cycle was cloned and sequenced.

Sixty-three clones were sequenced and six of them had sequences that were unique (called Tyr 1–6), each occurring various times (Fig. 1), and all arising from the same parental molecule. No primary sequence motif dominated the pool; three sequences (Tyr 1, Tyr 2, and Tyr 3), however, shared some sequence conservation, such as the four base motifs AUAC and UAGG at the 3′ half of the molecules (Fig. 1, nucleotides in dark grey boxes). In the same region, nucleotides conserved from the parental dopa2/c.1 sequence were found (Fig. 1, nucleotides in light grey boxes). The identity region corresponds to the SL 3 motif (stem and loop 3) of the dopa2/c.1 aptamer (see Fig. 8A).

Each unique sequence was tested for column affinity and for elution by free L-tyrosine. Clones Tyr 1, Tyr 2, and Tyr 3 effectively bound to both tyrosine-agarose and free tyrosine, whereas the unrelated clones Tyr 4, Tyr 5, and Tyr 6 appeared to be much less active and we did not characterized them further. The dissociation constant was determined by isocratic ligand elution for each of the active aptamers: Tyr 1 bound to free L-tyrosine with $K_d = 35$ μM, Tyr 2 and Tyr 3 with $K_d =$ 94 and 743 μM, respectively. Higher K_d values for tyrosine-agarose binding indicate that the selected aptamers favor the free tyrosine conformation over the matrix-bound conformation (Table 1).

TABLE 1. Dissociation constants (K_d) for agarose-bound tyrosine and tyrosine in solution. K_d values were determined by analytical affinity chromatography.

	K_d (μM)	
Aptamer	Tyr-agarose	Tyr
Tyr 1	755	35
Tyr 2	684	94
Tyr 3	3,200	743

RNA boundary mapping

Boundary experiments on partial alkaline hydrolysis products of Tyr 1, Tyr 2, and Tyr 3 RNAs allowed us to identify minimal 5′ and 3′ ends necessary for tyrosine binding. Figure 2 shows the least energy secondary structures of tyrosine-binding RNAs predicted by Mulfold (Zucker, 1989); experimental boundaries are indicated. The 5′ and 3′ boundaries map at different positions of the initially randomized region of aptamers Tyr 1 and Tyr 2, whereas Tyr 3-binding activity required part of the originally fixed 5′ and 3′ regions. The experimental boundaries were confirmed by the synthesis of truncated aptamers.

Specificity of tyrosine binding

To study binding specificity, tyrosine aptamers were tested for elution by tyrosine related molecules (Fig. 3).

Tyr 1b (aptamer derived from Tyr 1 with truncated 5′ and 3′ ends as indicated by boundary experiments; see Fig. 2) and Tyr 2 showed similar binding specificities, recognizing L-tyrosine, L-tryptophan, and L-dopa with the same affinity. The Tyr 1b-binding site was confirmed as being the most avid. For the Tyr 3 aptamer, L-tyrosine and L-dopa were better eluants than L-tryptophan. All three aptamers showed low affinity for L-phenylalanine. Figure 4 shows Tyr 1b isocratic affinity elution from tyrosine agarose: different eluant profiles illustrate binding specificity. The tyrosine-binding sites are highly L-enantio specific as indicated by the low affinity for D-tyrosine. Even though Tyr 1b was equally eluted by L-tyrosine, L-tryptophan, and L-dopa, a more detailed analysis of its binding specificity indicated a strong discrimination against side chains with a different conformation (see low affinity for O-phospho-L-tyrosine), as well as against an amino acid conformation versus an amine conformation (see tyramine and dopamine K_ds).

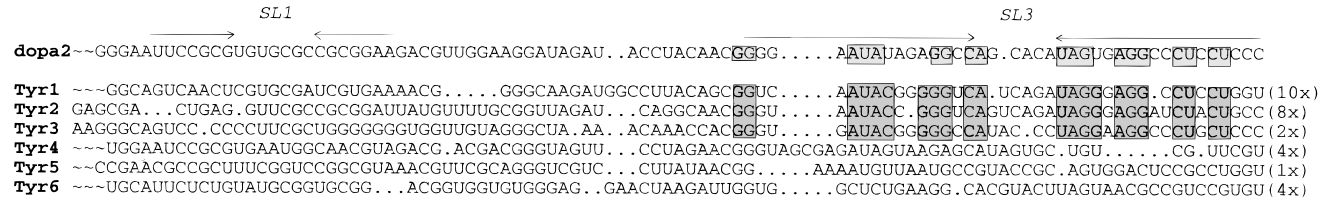


FIGURE 1. Sequences of unique clones from the final round of tyrosine selection. Multiple sequence alignment of initially randomized regions is reported. Clone identification numbers are given to the left of each sequence, and the number of identical clones is shown in parentheses on the right. Dopa2/c.1 sequence (dopa2) is reported above selected clones; SL1 and SL3 are minimal stem and loop structures necessary for dopamine binding. Sequence conservation of two or more nucleotides is indicated as boxed nucleotides: dark grey boxes refer to homologies among Tyr 1–3 aptamers; light grey boxes refer to homologies between Tyr 1–3 aptamers and dopa2/c.1.

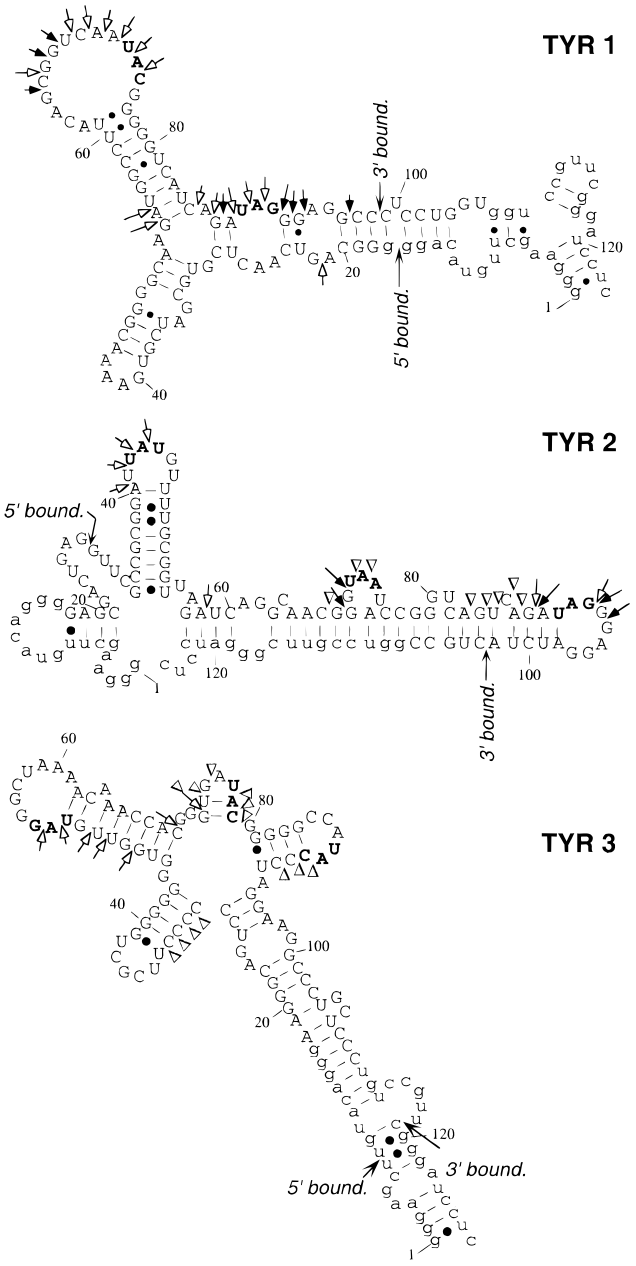


FIGURE 2. Summary of boundary mapping and enzymatic probing experiments. The positions of boundaries are reported on RNA secondary structures (5′ and 3′ bound.). Lowercase indicates PCR primer sites. Residues that are protected from nuclease digestion in the presence of tyrosine are indicated at the 3′ side of nucleotides on the RNA structures: open and closed arrows indicate S1 and T1 protections, respectively; open triangles indicate V1 protections. Nucleotides in bold are tyrosine and termination triplets.

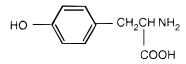
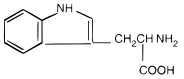
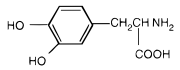
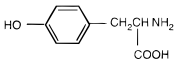
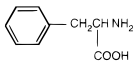
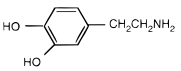
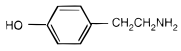
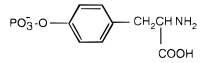
COMPOUND	STRUCTURE	Kd (μM)		
		Tyr 1b	Tyr 2	Tyr 3
L-TYROSINE		23 ±2	94±16	743
L-TRYPTOPHAN		22±1	73±9	1900
L-DOPA		22±3	82±12	728
D-TYROSINE		263±37	238	≥3200
L-PHENYLALANINE		183±26	119±38	≥ 3200
DOPAMINE		176±120	314	≥ 3200
TYRAMINE		188±76		
O-P-L-TYROSINE		238±6		

FIGURE 3. Specificity of tyrosine aptamers. Dissociation constants (K_d) for free ligands were determined by isocratic competitive affinity chromatography.

Recently, the isolation of an RNA aptamer able to bind L-tryptophan and L-phenylalanine with the same affinity has been reported (Zinnen & Yarus, 1995). Our analysis of tyrosine-aptamer binding properties indicates different features for our ligand-binding sites, which showed low specificity for the aromatic side chain of L-phenylalanine and high affinity for L-tyrosine, L-tryptophan, and L-dopa side chains.

RNA structural probing and identification of the binding site

To better define the tyrosine-binding site, RNA structural probing was performed using both single-strand (ss; T1 and S1) and double-strand (ds; V1) specific ribonucleases. By performing nuclease cleavage in the presence of L-tyrosine, we were able to identify positions in the RNA molecules that are protected from enzyme digestion by ligand binding, and to detect conformational changes induced by tyrosine-aptamer interaction. Figure 5 shows Tyr 1 enzymatic probing in the absence and in the presence of 1 mM L-tyrosine. The ss- and ds-nuclease digestion pattern essentially confirmed the secondary structure predicted by Mulfold (Fig. 2). After tyrosine binding no changes in the V1 cleavage pattern were observed. However, RNA–ligand interaction clearly induces modifications in the ss-specific nuclease digestion. The T1 digestion pattern indicates tyrosine induced protection of Gs 87, 91–93, and 96; strong tyrosine-induced protection of A21, G53, A54, C66, U69–A74, and C85–G90 was found after S1 digestion. Some of the protected residues belong to the consensus regions AUAC and UAGG in loop and bulge structures of the Tyr 1 RNA. Figure 2 shows a summary of Tyr 1, Tyr 2, and Tyr 3 enzymatic probing.

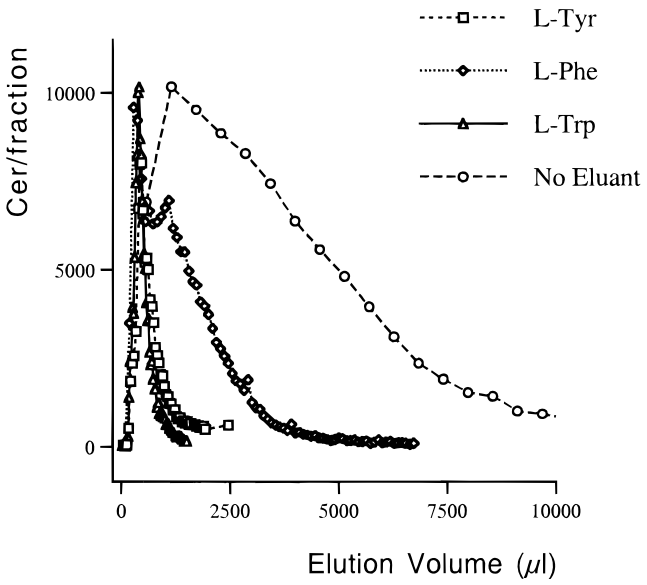


FIGURE 4. Tyr 1b isocratic affinity elution from tyrosine-agarose. The overlay of different eluant profiles is shown.

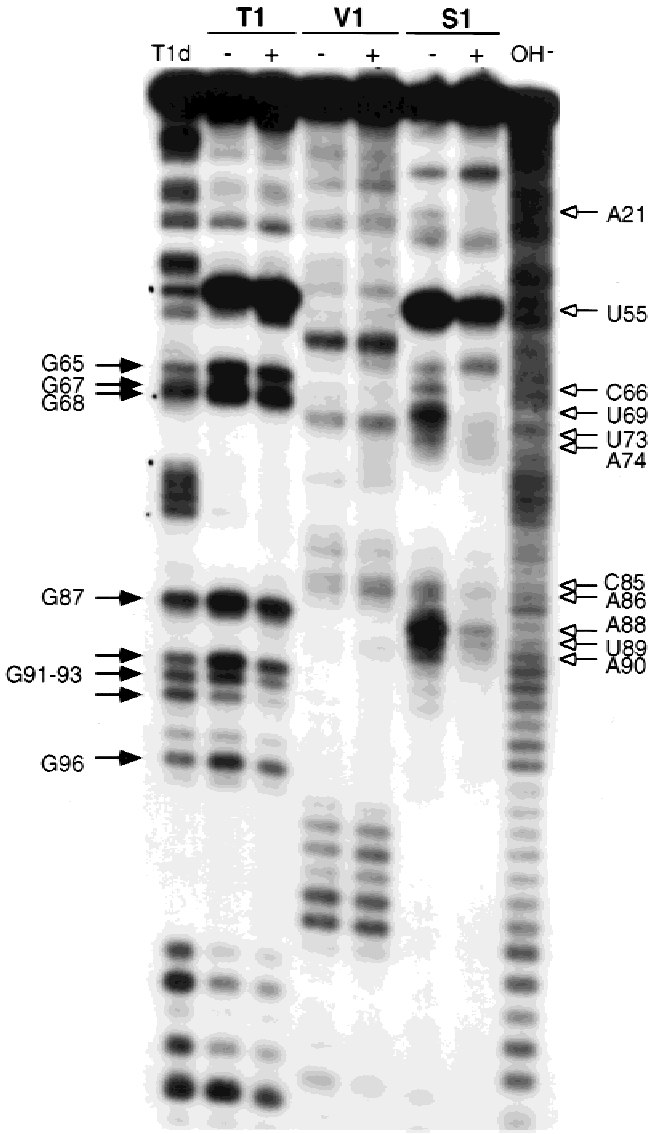


FIGURE 5. Enzymatic probing of the Tyr 1 aptamer. 3′ ^{32}P-end-labeled RNA was partially digested with RNase T1, V1, and S1 in the presence (+) or absence (−) of 1 mM tyrosine during nuclease digestion. Aliquots of enzymatic digestion were loaded onto a 14% polyacrylamide, 8 M urea gel along with alkaline hydrolysate (OH^-) and partial denaturing RNase T1 digest (T1d). Residues that are protected from nuclease digestion in the presence of tyrosine are indicated as open (S1) and closed (T1) arrows.

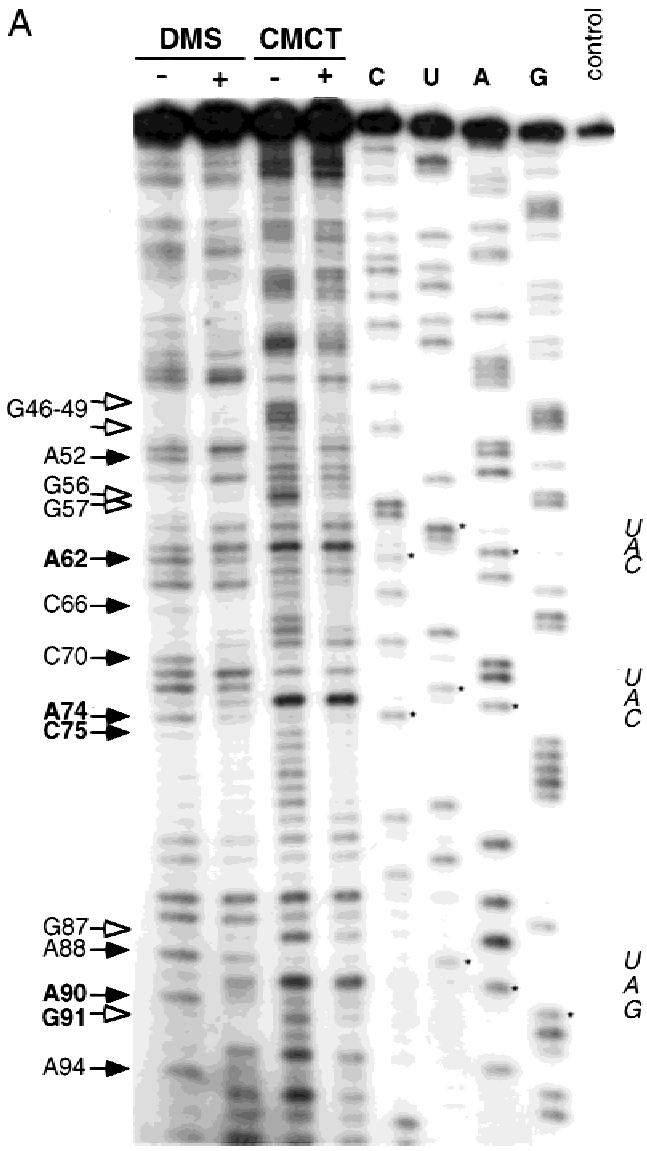
Defined positions in the Tyr 2 and Tyr 3 RNAs were protected from both ss- and ds-ribonuclease digestion after ligand binding. V1 cleavage of Tyr 2 RNA showed protection of bases U71 and A72, and A83–A87 upon ligand binding; tyrosine interfered with both S1 and T1 at positions A40–A43, G68 and G70, and G88–G93. V1 cleavage of Tyr 3 RNA indicated reduced susceptibility to nuclease attack of C29–C32, in the region from G73 to C79, and A89–C91. Sites of reduced susceptibility to S1 cleavage were found in the region from G47 to A53.

Tyrosine-binding sites map at different positions in the three selected aptamers. However, functional groups such as the UAGG or AUAC motifs, which are protected in all tyrosine aptamers, might be shared by the three RNAs.

To further define nucleotides that mediate ligand recognition, we performed chemical modification experiments on the Tyr 1 aptamer (Fig. 6A,B). Base-specific reactions were carried out with DMS and CMCT and modified bases were analyzed by primer extension. These experiments analyzed the sensitivity of N1 positions of A and G and the N3 positions of C and U to chemical modification. A number of bases were found to become strongly protected from modification upon tyrosine binding: C70, A74, C75, A88, and A90 were protected from DMS modification, and Gs 46–49, 56, 57, 87, and 91 from CMCT modification. Chemical probing experiments on Tyr 1 RNA showed similar protections upon L-tryptophan binding (data not shown).

In brief, enzymatic and chemical probing indicate that specific RNA structures are required for tyrosine recognition by the Tyr 1 aptamer. The protected residues are found in ss regions, loop, and internal bulges, some of which might be directly involved in contacting the ligand or in establishing new contacts induced by ligand binding. Interestingly, these structures are enriched on both tyrosine and termination codons. Protected sequences are enriched in UAN codons (bold nucleotides in Figs. 2 and 6, panels A and B): UAC (tyrosine codon) and UAG (termination/amber codon) in the Tyr 1; UAU (tyrosine) and both UAA (termination/ochre) and UAG in Tyr 2; and UAC and UAG in the Tyr 3 ligand-binding site.

Based on sequence data and on Tyr 1 structural probing, a smaller, 63-nt-long aptamer was synthesized



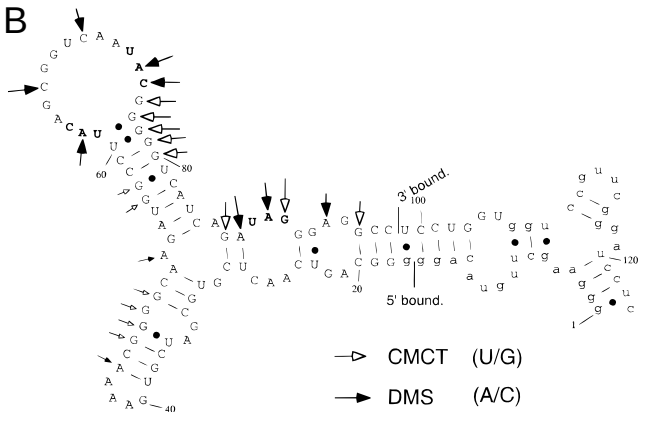


FIGURE 6. Chemical probing of the Tyr 1 aptamer. Positions with reduced sensitivity to chemicals in the presence of tyrosine (+) are indicated along the gel (**A**) and on the Tyr 1 secondary structure (**B**). Open arrows refer to residues protected from CMCT modification in the presence of tyrosine, closed arrows to residues protected from DMS modification. C, U, A, and G are dideoxy sequencing lanes. Bold nucleotides belong to UAN triplets, whose position along the sequencing reaction is shown.

(Fig. 7). The Tyr 1 minimal aptamer binds tyrosine agarose and is specifically eluted by 1 mM tyrosine just like the full-length aptamer, definitely proving that the ligand site resides within the protected regions.

Comparison between dopamine- and tyrosine-binding motifs

A comparative analysis of the parental dopamine-binding site and a tyrosine site is shown in Figure 8, which presents a schematic representation of dopa2/c.1 and Tyr 1b secondary structures.

After selection for tyrosine binding, some nucleotides of the parental aptamer were conserved and some new residues selected to perform the new activity. UAN triplets were already present in the parental sequence, with the exception of one UAC, generated through a U-to-C change. This new UAC triplet is present in all the three active aptamers (tyr 1–3) and it is absent in the tyr 4–6 sequences. This result suggests an essential role for the UAC triplet in tyrosine binding.

The dopamine-binding pocket is built up by a tertiary interaction between SL1 and SL3 (see Fig. 1): hydrogen bonding between complementary nucleotides allows the presentation of five residues that mediate ligand recognition (Mannironi et al., 1997). The overall structure of the amino acid aptamer is completely changed and the tyrosine-binding site (see minimal active sequence, Figs. 7 and 8), presents one loop and internal bulges where most of the protected residues reside. Different portions of the parental aptamer now contribute to the new activity: only half of SL1 is retained, whereas SL2, which is not necessary for dopamine binding, and a large portion of SL3 contribute to create the tyrosine-binding site.

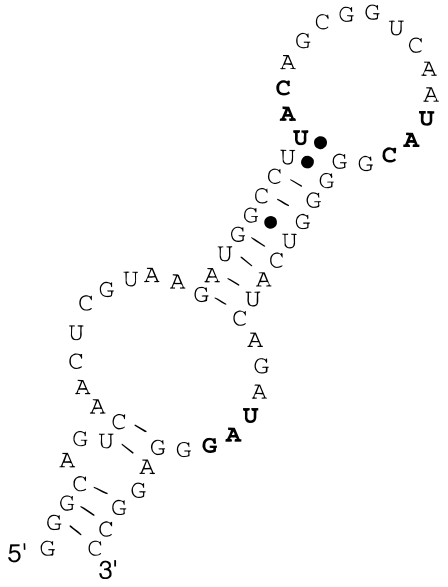


FIGURE 7. Tyr 1 minimal aptamer. UAN triplets are in bold.

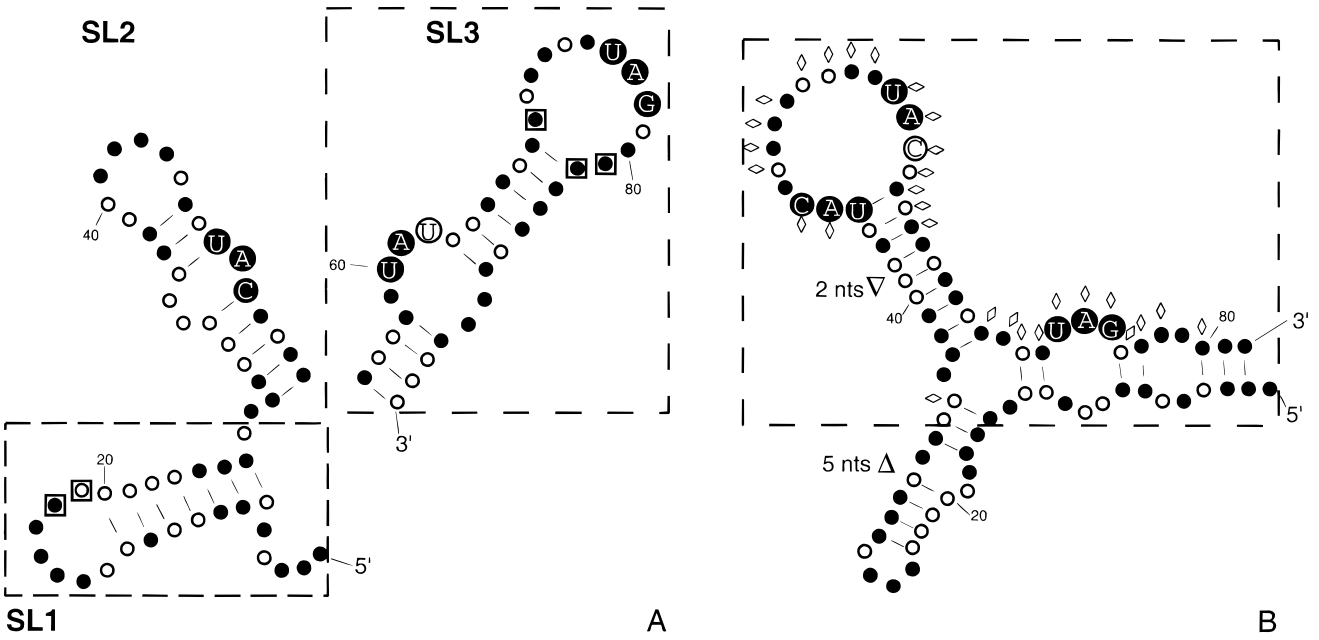


FIGURE 8. Comparison between dopamine and tyrosine aptamers. Secondary structures of the dopamine aptamer dopa2/c.1 (**A**) and of the tyrosine aptamer Tyr 1b (**B**) are shown. Only the UAN triplet sequences are specified in the structures. Black and white dots indicate, respectively, nucleotides conserved or changed in the tyrosine aptamer relative to the dopamine aptamer. ▽ and △ symbols indicate insertions and deletions in the Tyr 1 sequence. Dashed line frames indicate minimal active sequences for ligand binding. SL1–3 are stem and loop structures in the dopamine aptamer. Functional nucleotides important for dopamine recognition are boxed in **A**; protected nucleotides in the presence of tyrosine are indicated by diamonds in **B**.

In conclusion, the change in aptamer specificity might be ascribed both to specific changes and to a different usage of conserved sequence motifs, rather than to a minor alteration of the parental binding site.

DISCUSSION

RNA motifs with binding affinity for the free amino acid tyrosine were evolved from a pool derived from the partial randomization of a dopamine-binding sequence. Three independent aptamers were repetitively isolated and Tyr 1, the aptamer presenting the highest affinity for the amino acid, was characterized in detail in terms of binding specificity and binding-site structure.

Tyr 1 binds L-tyrosine with a $K_d = 35\ \mu$M. Competitive affinity chromatography performed with different ligands suggests high L-enantio specificity for all the three tyrosine aptamers. Tyr 1 and Tyr 2 bind L-tyrosine, L-dopa, and L-tryptophan with similar affinities. The binding of the three aromatic ligands might involve the recognition of their overall shape and stacking interactions. Another possibility is that the ring N-H of tryptophan is somehow equivalent to the ring -OH of tyrosine and L-dopa, with no large ring affinity implied. Our binding site recognizes L-dopa with the same affinity for tyrosine. The tyrosine-binding site, because it is derived from a doped dopamine-binding site, could have some remembrance of the modalities of recognition of dopamine and, as a consequence, could accommodate the L-dopa -OH at position 3 of the benzene ring.

RNAs with affinity for the side chains of tryptophan and phenylalanine have been previously described (Zinnen & Yarus, 1995). The structure of the tryptophan/phenylalanine-binding site is not available; however, the low affinity of our tyrosine aptamers for phenylalanine indicates new features for our sites.

Preliminary results on Tyr 1 minimal aptamer structure analysis suggest that tertiary interactions between ss regions of the molecule might be involved in binding-pocket formation, in contrast with the small asymmetric loop described for the other amino acid-binding sites.

The dopa2/c.1 RNA (Mannironi et al., 1997) was chosen as a source of highly mutated sequences for tyrosine selection because of the structural similarity between the two ligands. We found that changes in the target molecule result in a dramatic change of the aptamer structure, with the loss of the framework described for the dopamine-binding site. Yet, sequence motifs of the former binding site (in SL2 and SL3) have been retained and adapted to the new role in tyrosine binding.

Structural probing in the presence of tyrosine allowed us to localize sequences directly involved in the formation of ligand-binding pocket. Tyr 1 functional sequences include tyrosine UAC codons and an amber UAG termination codon. Functional coding triplets, both

tyrosine codons (UAU and UAC) and termination codons (UAG and UAA) are also found in the Tyr 2 and Tyr 3 sequences protected from enzymatic probing upon ligand binding. Thus, RNA–tyrosine complexes involve coding and termination triplets that belong to the same box of the genetic code of tyrosine codons. UAN triplets appear to be enriched at the level of the binding site; 26% of the site nucleotides are members of the coding triplets (22/84) versus 11% triplet in outside, nonsite nucleotides. Perhaps all UAN codons were initially assigned to tyrosine, rather than being partially used as nonsense. No UGA triplets exist in the tyrosine sites.

The comparison between dopamine and tyrosine aptamers (Figs. 1 and 8) shows that both tyrosine and one amber codons were already present in the dopa2/c.1 dopamine aptamer sequence. The upstream UAC is already present in SL2, a region found to be unused for dopamine binding; the UAG is located, in the parental aptamer, in a region that varies extremely among the dopamine aptamers in terms of nucleotide composition (Mannironi et al., 1997). Tyrosine coding triplets are not involved in dopamine recognition; instead, they are selected at the level of the tyrosine-binding site, where they appear to be involved in the formation of the tyrosine-binding pocket.

Amino acid sites in which the corresponding coding triplets have been previously found are the natural and in vitro selected arginine sites and isoleucine sites (Connell et al., 1993; Connell & Yarus, 1994; Burgstaller et al., 1995; Majerfeld & Yarus, 1998; Yarus, 1998). A statistical association between binding sites and codons has been shown for all the sites currently characterized (Yarus, 2000). The implications for the stereochemical theory of the origin of the genetic code have been discussed (Yarus, 1998; Knight & Landweber, 1998; Knight et al., 1999; Yarus, 2000). Further analysis of additional amino acid aptamers will contribute to the elucidation of the role of codons in RNA–amino acid recognition.

MATERIALS AND METHODS

In vitro selection

The RNA pool with a random insert of 93 nt was obtained by in vitro transcription of a doped dopamine aptamer (Mannironi et al., 1997). PCR primers include a T7 promoter and *Hin*dIII restriction site (upstream primer: 5′-TAA TAC GAC TCA CTA TAG GGA AGC TTG TAC AGG G) and *Bam*H1 (downstream primer: 5′-GGT CCG TTC GGG ATC CTC A). The random RNA pool contained 3×10^{13} different molecules. Tyrosine selection was done in 0.2 mL affinity columns, containing 10 mM L-tyrosine linked through its amino group to cyanogen bromide-activated agarose. The selection buffer contained 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM $MgCl_2$, and the elution was done in column buffer containing 1 mM L-tyrosine. At cycles 7 and 8 and from cycle 10 to cycle 13 tyrosine selection was preceded by a counter selection on sepharose columns to remove RNA with affinity for the resin.

^{32}P-labeled RNA dissolved in water was denatured for 5 min at 65 °C, adjusted to column buffer, and cooled to room temperature for 10 min. The RNA was then applied to the column and allowed to bind to the column ligand for 10 min. Increasing volumes of washes (from 6 to 20 column volumes) preceded the affinity elution. The RNA eluted by L-tyrosine was precipitated, amplified, cloned, and sequenced as previously described (Mannironi et al., 1997).

Boundary mapping

The minimal sequence required for binding to tyrosine column and elution with tyrosine was determined as follows: the RNA was labeled at the 3′ and 5′ ends with ^{32}P, partially hydrolyzed under alkaline conditions (Pan & Uhlenbeck, 1992), and precipitated with ethanol. Selection of digestion products was performed under in vitro selection conditions eluting bound RNAs with 1 mM tyrosine. Aliquots of the collected fractions were loaded onto 8% polyacrylamide gel containing 8 M urea for autoradiography.

Determination of dissociation constants

RNA dissociation constants (K_d) for tyrosine agarose and free ligands were determined by isocratic competitive affinity chromatography (Connell et al., 1993; Majerfeld & Yarus, 1998). From 15 to 20 pmol of internally ^{32}P-labeled RNA was applied to the affinity matrix after denaturation and renaturation in column buffer and 0.2 mM eluant when present.

Enzymatic probing

RNA transcripts obtained by in vitro transcription were 5′- and 3′-^{32}P-end labeled. RNAse T1 (0.1 U/μL), V1 (0.024 U/μL), and S1 (10 U/μL) digestions were performed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM $MgCl_2$ as previously described (Mannironi et al., 1997). Reaction times ranged from 30 s to 7 min at 37 °C (T1 and S1) and 22 °C (V1). For the study of nuclease cleavage in the presence of the ligand, 1 mM tyrosine was dissolved in the reaction buffer.

Chemical modification

Chemical reaction with DMS was performed in 25 mM Na-HEPES, pH 7.4, 40 mM NaCl, and 5 mM $MgCl_2$. The CMCT reaction buffer was 50 mM Na Borate, pH 8, 40 mM NaCl, and 5 mM $MgCl_2$. One picomole of Tyr 1 RNA was denatured at 65 °C for 5 min in the reaction buffer, cooled to room temperature for 20 min, and modified at room temperature with DMS (1/600 dilution, v/v) for 5 min or with CMCT (final concentration 10 mg/mL) for 10 min. Reactions were stopped by precipitating the RNA at −70 °C after addition of 10 μg of bulk yeast tRNA (Sigma), 60 μL of 0.5 M sodium acetate, and 300 μL of ethanol. RNA pellets were washed with 70% ethanol prior to drying.

Detection of modified bases was accomplished by extension with reverse transcriptase of the 5′-end-labeled down-

stream primer. Reverse transcription was performed as described (Costa et al., 1997) with minor modifications: elongation and sequencing reactions were performed at 52 °C and 46 °C, respectively; dNTPs concentration in the sequencing reaction was 0.25 mM; and the reverse transcriptase used was the SuperScript II RNase H reverse transcriptase (Gibco-BRL).

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