RNA hairpin aptamers specific for the trans-activation-responsive (TAR) RNA element of human immunodeficiency virus type 1 were identified by in vitro selection (Duongé, F., and Toulmé, J. J. (1999) RNA 5, 1605–1614). The high affinity sequences selected at physiological magnesium concentration (3 mM) were shown to form a loop-loop complex with the targeted TAR RNA. The stability of this complex depends on the aptamer loop closing “GA pair” as characterized by preliminary electrophoretic mobility shift assays. Thermal denaturation monitored by UV-absorption spectroscopy and binding kinetics determined by surface plasmon resonance show that the GA pair is crucial for the formation of the TAR-RNA aptamer complex. Both thermal denaturation and surface plasmon resonance experiments show that any other “pairs” leads to complexes whose stability decreases in the order AG > GG > GU > AA > GC > UA > CA, CU. The binding kinetics indicate that stability is controlled by the off-rate rather than by the on-rate. Comparison with the complex formed with the TAR* hairpin, a rationally designed TAR RNA ligand (Chang, K. Y., and Tinoco, I. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8705–8709), demonstrates that the GA pair is a key determinant which accounts for the 50-fold increased stability of the TAR-aptamer complex (Kd = 2.0 nM) over the TAR-TAR* one (Kd = 92.5 nM) at physiological concentration of magnesium. Replacement of the wild-type GC pair next to the loop of RNA I by a GA pair stabilizes the RNA I’-RNA II’ loop-loop complex derived from the one involved in the control of the ColE1 plasmid replication. Thus, the GA pair might be the preferred one for stable loop-loop interactions.

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† The abbreviations used are: HIV-1, human immunodeficiency virus type 1; nt, nucleotide(s); SPR, surface plasmon resonance; EMSA, electrophoretic mobility shift assay; RU, resonance unit(s); TAR, trans-activation-responsive.
interactions other than loop complementarity are crucial for stability. Indeed, several mutations of the GA pair that closes the loop of the identified RNA aptamers decrease the stability of the TAR-aptamer complex, as shown by preliminary electrophoretic mobility shift assays (EMSA).

In the work presented herein, the role of this GA pair was investigated at physiological concentration of magnesium by systematically mutating the loop closing pair of the aptamer. The effects of these mutations on the stability of the TAR RNA-aptamer complex were analyzed by thermal denaturation monitored by UV-absorption spectroscopy (26, 27). The binding kinetics were determined by using surface plasmon resonance (SPR). This physical phenomenon is used to follow in real time the interaction between a molecule in a continuous flow and an immobilized one (28). Numerous studies have been published on protein-protein interactions, protein-ligand interactions, and protein-nucleic acid interactions, but a much reduced number of investigations of nucleic acid-nucleic acid interactions are available (29–31). As a matter of fact, no RNA-RNA complexes have been analyzed up to now.

Our results demonstrate that the aptamer loop closing GA pair is crucial for the stability of the TAR-RNA aptamer complex once formed. This likely explains the higher stability of the loop-loop complex formed by TAR with the aptamer, at physiological magnesium concentration, compared with the one formed with the rationally designed hairpin, TAR*, whose loop is closed by a UA pair (32). The increased stability of a loop-loop complex formed with the rationally designed hairpin, TAR*, whose loop is closed by a UA pair is crucial for the stability of the TAR-RNA aptamer complex (33). The increased stability of a loop-loop complex formed between RNA II* and a RNA I* mutant in which the GC pair next to loop was replaced by GA suggests that closing GA pair could be preferred for kissing complexes.

MATERIALS AND METHODS

Oligonucleotides—All RNA molecules including the biotinylated TAR RNA were synthesized on an Expedite 8908 synthesizer and purified by electrophoresis on denaturing polyacrylamide gels. The pure samples were desalted on Sephadex G-25 spin columns. To avoid repeated thawing and freezing of the stock solutions, the samples were aliquoted at a volume and a concentration suitable for each experiment and stored at −20 °C. Before the experiments, each RNA sample used was heated at 95 °C during 1 min and then put on ice for 10 min to avoid the formation of intermolecular complexes.

Thermal Denaturation of RNA Complexes—Thermal denaturation of RNA complexes in 20 mM sodium cacodylate buffer, pH 7.3, at 20 °C, with 140 mM potassium chloride, 20 mM sodium chloride and 3 mM magnesium chloride (R buffer) was monitored on a Cary 1 spectrophotometer interfaced with a Peltier effect device that controls temperature within ±0.1 °C. Denaturation of the samples was achieved by increasing the temperature at 0.4 °C/min from 5 °C to 90 °C and was followed at 260 nm. A cuvette that contained R buffer was used as the reference. Except cacodylate, which replaced the temperature-sensitive HEPES, the buffer used for these thermal denaturation experiments was equivalent to the one used during the in vitro selection process (25). As the absorbance of the TAR RNA at 260 nm is too large to allow accurate monitoring of the absorption change resulting from the denaturation of the bimolecular complex between the HIV-1 RNA and the RNA aptamer, the experiments were carried on with miniTAR, a 27-mer oligonucleotide, instead of the entire TAR hairpin, which is 59 nt. RNA samples were prepared at 1 μM final concentration in the mixture. They were mixed at room temperature and allowed to interact 30 min before cooling down to 5 °C. The experiment then started after 1 h at this temperature.

The enthalpy change, ΔH, for the formation of the bimolecular complex, was deduced from the total concentration dependence of the Tm according to Equation 1.

\[
\Delta H_{\text{binding}} = \frac{R}{\Delta T_m} \ln[Mg^{2+}]_{\text{final}} + \frac{\Delta S - R\ln 4}{\Delta H}
\]  

(Eq. 1)

The number of magnesium ions that bind upon formation of the complex, ΔMg²⁺, was determined from the ion concentration dependence of the melting temperature, Tm, according to Equation 2.

\[
\frac{\Delta T_m}{\Delta \ln[Mg^{2+}]_{\text{final}}} = \frac{\Delta \ln[Mg^{2+}]_{\text{final}}}{\Delta H} = -\frac{\Delta S - R\ln 4}{\Delta H}
\]  

(Eq. 2)

Surface Plasmon Resonance Kinetic Measurements—SPR experiments were performed on a BIAcore 2000 apparatus (Biacore AB, Sweden) running with the BIAcore 2.1 software. Biotinylated TAR RNA, 59 nt long, was immobilized on CM5 sensorscoth coated with streptavidin according to the procedure described in the BIA applications handbook. In these conditions, 5000 resonance units (RU) of streptavidin (Sigma), equivalent to 5 ng/mm², were immobilized on the chip which subsequently was allowed to equilibrate at 23 °C, the temperature of the in vitro selection, in the selection buffer: 20 mM HEPES, pH 7.3, at 20 °C containing 20 mM sodium acetate, 140 mM potassium acetate, and 3 mM magnesium acetate (R buffer). Biotinylated TAR RNA (10–50 nm) was prepared in this buffer and then injected at a flow rate of 5 μl/min. The injection was stopped as soon as 500–600 RU of bound TAR RNA was reached. This amount was shown to be appropriate to keep the pseudo-first order kinetic condition and to allow good reliability of recorded sensograms (RU versus time) in terms of signal to noise ratio. One noncoated or one streptavidin-coated flow-cell was used to check for nonspecific binding of RNA aptamers. The signals from these control channels served as base lines and were subtracted to the RU change observed when an injected RNA aptamer interacts with the immobilized TAR RNA. A nonreducing hairpin from the hepatitis C virus RNA, was used as a negative control (33). The sensor chip surface was successfully regenerated with three 5-μl pulses of 25% formamide, followed by one 5-μl pulse of distilled water and finally one 10-μl pulse of R buffer.

Nonlinear regression analysis of single sensograms at five concentrations, at least, of injected RNAs was used to determine the kinetic parameters of the complex formation. The data were analyzed with the BIA evaluation 2.2.4 software, assuming a pseudo-first order model, according to Equations 3–5, for the association and dissociation phases, respectively, where R is the signal response, Rmax the maximum response level, C the molar concentration of the injected RNA molecule, k on the association rate constant, and k off the dissociation rate constant.

\[
\frac{dR}{dt} = h_{\text{on}}C(R_{\text{max}} - R) - h_{\text{off}}R
\]  

(Eq. 3)

\[
\frac{dR}{dt} = -h_{\text{off}}R
\]  

(Eq. 4)

To check for self-consistency of data, h on derived from nonlinear analysis, was plotted as a function of the RNA concentration according to Equation 5.

\[
h_{\text{on}} = k_{\text{on}}C + k_{\text{off}}
\]  

(Eq. 5)

RESULTS

The RNA molecules used for this study are shown in Fig. 1. MiniTAR (27 nt long), the upper half part of TAR, was shown to be the minimal domain necessary and sufficient for responsiveness in vitro (34). It interacts with the anti-TAR selected aptamers without changes in the affinity compared with the full-length TAR (35). R-0624(GA) is the aptamer of highest affinity identified by in vitro selection against TAR, with the consensus motif 5′-GUCCCGAGA-3′, LR-06 9, in the apical loop. The six central bases of the consensus sequence are complementary to the entire TAR loop. TAR is a hairpin rationally designed to interact with TAR (32). Its loop is fully complementary to the TAR one. RNA I* and RNA II* are two structured RNAs derived from the two RNA transcripts, RNA I and RNA II, involved in the control of the ColEl plasmid regulation. The sequences of RNA I* and RNA II* were modified in the stem to avoid the formation of an extended duplex, as seen with the biological RNA once the kissing complex is formed (6).

Detection of the Loop-Loop interaction by Thermal Denaturation—The derivative of UV melting curves of miniTAR with R-0624(GA) aptamer, with TAR* RNA and with LR-06 9 are reported in Fig. 2. The melting profiles obtained with mixtures of miniTAR and R-0624(GA) RNA at two different concentrations in R buffer display two transitions (Fig. 2A). Only one transition is observed for the melting profiles of the RNA hairpin...
pinnal. On diluting the miniTAR and R-0624(GA) aptamer mixture 4-fold (from 2 μM to 0.5 μM), the \( T_m \) (the maximum of the derivative plots) of the lower transition decreases from 47.5 °C to 40 °C as expected for a bimolecular complex, whereas the \( T_m \) of the higher transition remains unchanged and thus can be ascribed to the melting of the RNAs alone.

We then compared the stability of the miniTAR and R-0624(GA) aptamer mixture to that of two reference complexes: miniTAR with either TAR* RNA or LR-06 8 (Fig. 2B). Under the ionic conditions used for the in vitro selection, \( T_m \) for the complex with TAR* is equal to 30.7 °C (Table I), i.e. 16.8 °C below that of the miniTAR-R-0624(GA) complex. Finally, \( T_m \) for the complex with LR-06 8, the 8-mer RNA 5'GUCCGAGA' 3' of the consensus motif of the aptamers, is equal to 20.3 °C. Clearly, interaction of miniTAR with the aptamer gives rise to the most stable bimolecular complex. On one hand, the higher stability of the TAR-aptamer complex over that of the 8-mer RNA can be ascribed to the hairpin structure of the ligand as previously demonstrated (36). On the other hand, the comparison between TAR* and the selected aptamer strongly suggests that the sequence outside the loop plays a crucial role in the extra stability displayed by TAR-aptamer complexes compared with the TAR-TAR* one.

The stability of RNA structures and complexes is well known to depend on magnesium ion. We then wanted to address the question whether the differential behavior between TAR* and the aptamer would originate in the number of associated Mg\(^{2+}\) ions. This can be achieved by measuring the variation of \( T_m \) as a function of the Mg\(^{2+}\) concentration in solution at 23 °C, for both complexes can be easily determined. \( K_d \), the equilibrium dissociation constant, was determined from the slope of the concentration dependence of the \( T_m \) (Fig. 3), according to Equation 1. In these experiments, the concentration of Mg\(^{2+}\) was decreased from 3 mM in R' buffer to 1 and 0.1 mM, for the miniTAR-TAR* and miniTAR-aptamer complexes, respectively, to accurately measure the \( T_m \) values of bimolecular complexes in the total RNA concentration range chosen, 0.5–16 μM, with no interference with the melting of the hairpins alone. Under these conditions, \( \Delta H \) is equal to \(-42.8 \pm 1.4\) kcal/mol and \(-39.2 \pm 1.3\) kcal/mol for miniTAR-R-0624(GA) and miniTAR-TAR* complexes, respectively. The variation of \( T_m \) as a function of the Mg\(^{2+}\) concentration is presented in Fig. 4. For both complexes, linearity of 1/\( T_m \) versus ln[Mg\(^{2+}\)] plots indicates a site binding mode of the magnesium ion. The number of ions, \( \Delta \)Mg\(^{2+}\), which bind was deduced according to Equation 2 using the enthalpy change. This gives \( \Delta \)Mg\(^{2+}\) = 1.7 ± 0.1 and 1.4 ± 0.1 for the complexes formed with R-0624(GA) and TAR*, respectively. It demonstrates that the different stability of these complexes does not originate in a difference in the number of magnesium ions that each complex binds. From this \( \Delta H \) and the \( T_m \) values measured at 3 mM magnesium (Table I), the equilibrium dissociation constant, \( K_d \), in solution at 23 °C, for both complexes can be easily determined. \( K_d \), under the in vitro selection conditions, is equal to 2.0 ± 0.4 nM and 92.5 ± 5.2 nM for miniTAR-R-0624(GA) and miniTAR-TAR* complexes, respectively. Clearly, under this condition, the miniTAR-aptamer complex is more stable than the one formed with TAR*, the rationally designed RNA.

The base pair closing the loop was a marked difference between TAR* and the aptamer molecule. Then, in a second set of

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**Fig. 1. Secondary structure of RNAs.** The TAR RNA (top right) was used as a target for R-0624 aptamer derivatives or TAR* (top left). The arrows indicate the 5' and 3' ends of truncated molecules. The loop closing pair, which was mutated in the aptamer, is in bold and italic. RNA 1' and II' are derived from structures (RNA I and RNA II) involved in ColE1 replication. In RNA 1', the GC pair in bold and italic was replaced by GA.

**Fig. 2. Second derivative UV (260 nm) melting curves of complexes with miniTAR.** The experiments were performed with 1 μM amount of each RNA in buffer R'. A, complex with R-0624(GA) (thin line), 4-fold diluted complex with R-0624(GA) (bold line), miniTAR alone (bold dotted line), R-0624(GA) alone (thin dotted line). B, complex with R-0624(GA) (thin line), with TAR* (bold dotted line) or with LR-06 8 (bold line).
Surface Plasmon Resonance Detection of RNA Complexes—

SPR was used to follow, in real time, the interaction of the immobilized full-length TAR RNA on streptavidin-coated sensorchip with various RNA hairpins. Sensorgrams, obtained when R-0624(GA) or TAR* analytes were injected over the sensorchip surface at two different concentrations of magnesium ion, are reported in Fig. 6. In all cases, as expected for a pseudo-first order model, the dissociation phase does not show significant dependence on the concentration of the injected analyte whereas the association phase increases with it. Furthermore, these kinetics fulfill pseudo-first order conditions as checked from linearity of plots of the observed rate constant, \( k_{\text{obs}} \) versus the analyte concentration (insets). R-0624(GA) aptamer binds to TAR RNA at either 3 or 10 mM magnesium, as shown. Linear fits were calculated according to Equation 2.

![Image](315x331 to 547x510)

**FIG. 3. Dependence of \( T_m \) on the total RNA concentration for miniTAR complexes.** The experiments with TAR* (○) were performed at 1 mM Mg\(^{2+}\). Those with R-0624(GA) (■) were performed at 0.1 mM Mg\(^{2+}\). Linear fits were calculated according to Equation 1.

![Image](52x62 to 294x452)

**TABLE I**

Melting temperature, \( T_m \), of RNA complexes and RNAs alone

The experiments with miniTAR either with TAR*, the RNA aptamers or the truncated versions were performed in \( R' \) buffer at 1 \( \mu \)M each RNA, those with RNA I' and RNAII' in \( R' \) buffer + 7 mM Mg\(^{2+}\) at 2 \( \mu \)M each RNA. \( T_m \) is the average and standard deviation of two or three experiments.

| RNA       | MiniTAR-RNA complex | RNA alone |
|-----------|---------------------|-----------|
| \( ^\circ \C \) | \( ^\circ \C \) | \( ^\circ \C \) |
| R-0624(GA) | 47.3 ± 0.3          | 70.8 ± 0.7 |
| R-0624(AG) | 42.8 ± 0.4          | 70.7 ± 0.7 |
| R-0624(GG) | 37.0 ± 0.0          | 72.3 ± 0.0 |
| R-0624(GU) | 32.9 ± 0.1          | 71.4 ± 0.0 |
| R-0624(AA) | 31.5 ± 0.6          | 65.1 ± 0.6 |
| R-0624(GC) | 31.4 ± 0.6          | 81.5 ± 0.0 |
| R-0624(UA) | 29.9 ± 0.6          | 75.5 ± 0.5 |
| R-0624(CA) | 21.0 ± 0.0          | 70.3 ± 0.4 |
| R-0624(CU) | 16.8 ± 1.1          | 73.0 ± 0.1 |
| Tar*      | 30.7 ± 0.6          | 66.1 ± 0.8 |
| R-0624(UA) | 31.4 ± 0.5          | 62.9 ± 0.4 |
| LR-06     | 20.3 ± 0.4          | ND\(^a\) |
| MiniTAR   | 69.9 ± 0.2          |           |

**FIG. 4. Dependence of \( T_m \) on the concentration of Mg\(^{2+}\) for miniTAR complexes.** The experiments were performed using 1 \( \mu \)M amount of each RNA. Complex with TAR* (○) or with R-0624(GA) (■) is shown. Linear fits were calculated according to Equation 2.

**FIG. 5. Second derivative UV (260 nm) melting curves of complexes with miniTAR.** Figure shows complex with R-0624(GA) (thin line), with R-0624(AA) (dotted line), or with R-0624(CU) (bold line).
thermodynamically favorable for loop-loop interactions and
mid—
In an attempt to establish whether a closing GA pair is
inversion, which is equivalent to the wild-type aptamer in
terms of stability, with a
by the off-rate, which increases when the complex is destabi-
ysis shows that the equilibrium constant is mainly controlled
iments, whereas the on-rate shows limited variation.
The effects of the other mutations are in between. Closer anal-
such a degree that the rate constants cannot be determined.
CU and CA mutations destabilize the TAR-aptamer complex to
buffer R
1
D
3m M and 10 mM Mg2
mM magnesium).
The experiments were performed either in R buffer or in R buffer
7m M Mg2
7m M Mg2
R
R
R
R
in buffer R (3 mM magnesium). Mutants of
injected, and RU variations that resulted from complex formation were deduced from direct fitting of these plots according to Equations 3 and 4. Inset, concentration dependence of the observed rate constant
off, for bimolecular com-
Kd

derived from electrophoretic mobility shift assays (Ducongé & Toulmé, 1999).

| Buffer | RNA       | k_on  | k_off | Kd(BIAcore) | Kd(EMSA) |
|--------|-----------|-------|-------|-------------|----------|
|        | × 10^6 M^-1 s^-1 | × 10^-2 M^-1 s^-1 | nM   | nM          |
| R      | R-0624    | 6.3  ± 0.6 | 1.1 ± 0.1 | 17 ± 3 20   |          |
|        | TAR*      | ND    | ND    | ND          | >1000    |
| R + 7 mM Mg2+ | R-0624 | 17 ± 1 | 0.93 ± 0.1 | 5.4 ± 0.8 6 |          |
|        | TAR*      | 38 ± 7 | 5.2 ± 0.1 | 14.5 ± 3 10 |          |

* ND, not determined.

step, as expected for larger screening of electrostatic repulsions.
The role of the GA closing pair of R-0624 RNA was further examined by SPR in R buffer (3 mM magnesium). Mutants of the closing pair were injected, and RU variations that resulted from complex formation were monitored as a function of time. The rate constants, k_on and k_off, deduced from nonlinear regression analysis of sensorgrams are listed in Table III as well as the equilibrium dissociation constant, K_d. Except for the AG inversion, which is equivalent to the wild-type aptamer in terms of stability, with a K_d of about 20 nM, all other mutations have a negative effect on the stability of the TAR-RNA complex. CU and CA mutations destabilize the TAR-aptamer complex to such a degree that the rate constants cannot be determined. The effects of the other mutations are in between. Closer analysis shows that the equilibrium constant is mainly controlled by the off-rate, which increases when the complex is destabilized, whereas the on-rate shows limited variation.

TABLE II Effects of mutations of the loop closing GA pair of the aptamer on equilibrium and rate constants for TAR binding

| RNA       | k_on  | k_off | Kd(BIAcore) | Kd(EMSA) |
|-----------|-------|-------|-------------|----------|
| R-0624(GA)| 6.3  ± 0.6 | 1.1 ± 0.1 | 17 ± 3 20   |          |
| R-0624(AG)| 6.8  ± 0.9 | 1.5 ± 0.1 | 22 ± 2 32   | ± 9      |
| R-0624(GG)| 7.9  ± 0.4 | 4.1 ± 0.1 | 52 ± 2 63   | ± 15     |
| R-0624(GU)| 8.5  ± 1.4 | 9.1 ± 0.2 | 107 ± 14 133 ± 20 |
| R-0624(AA)| 5.7  ± 0.5 | 6.4 ± 0.1 | 122 ± 12 340 ± 80 |
| R-0624(GC)| 10.5 ± 0.3 | 18.5 ± 0.8 | 167 ± 22 264 ± 130 |
| R-0624(UA)| 6.2  ± 0.3 | 13.0 ± 0.1 | 206 ± 1 440 ± 130 |
| R-0624(UC)| ND    | ND    | ND          | >1000    |
| R-0624(CU)| ND    | ND    | ND          | >1000    |

* ND, not determined.

Thus might constitute a rule, we replaced the GC pair next to the 7-nm-long loop of RNA I’ by a GA pair (Fig. 1). The seven central bases of the new loop, closed by a GA pair, are complementarily to the entire RNA II’. The effect of this mutation was analyzed by thermal denaturation. The results obtained for the complex with the GA mutant and the unmodified RNA I’ are reported in Fig. 7. A clear transition with a T_m equal to 23.8 °C is seen for the complex with the GA mutant, whereas no significant transition is observed for the wild-type complex. This transition is concentration-dependent (data not shown), as expected for a bimolecular process. The transitions above 60 °C are ascribed to the melting of the RNAs alone (concentration-independent). Interestingly, this new transition is observed despite the lower stability of the GA RNA I’ mutant compared with the wild-type molecule. This is actually in agreement with the removal of a GC pair. T_m is equal to 76 °C and 70 °C for the latter and the former, respectively (Table I). This indicates that the GA pair is a key structural determinant for the stability of the RNA I’-RNA II’ complex as well.

FIG. 6. Sensorgrams of TAR-R-0624(GA) and TAR-TAR* complexes at 3 mM and 10 mM Mg2+ complexes at 3 mM and 10 mM Mg2+. Increasing concentrations of RNAs as indicated by the arrow were injected on the TAR-functionalized sensorchip. Elementary rate constants, k_on and k_off, for bimolecular complex formation were deduced from direct fitting of these plots according to Equations 3 and 4. Inset, concentration dependence of the observed rate constant k_off A, injected R-0624(GA) in buffer R (3 mM magnesium). B, injected R-0624(GA) in buffer R + 7 mM Mg2+. C, injected TAR* in buffer R. D, injected TAR* in buffer R + 7 mM Mg2+.
Closing GA Pair in Loop-Loop RNA Complexes

The experiments were performed in R buffer + 7 mM Mg<sup>2+</sup> at 2 μM of each RNA. Figure shows RNA I(GC)-RNA II complex (thin line), RNA I(GA) mutant-RNA II complex (bold line).

**DISCUSSION**

*In vitro* selection against the trans-activation-responsive RNA of HIV-1 identified RNA hairpin aptamers, which form kissing complexes with the targeted RNA at physiological concentration of magnesium (3 mM) (25). Together with loop complementary, the loop closing GA pair of the aptamer of highest affinity, R-0624(GA), is critical for the stability of such complexes, as shown by electrophoretic mobility shift assays. In this work, the contribution of the GA pair to the thermodynamics and the kinetics of the loop-loop interaction was investigated by using UV-absorption spectroscopy and surface plasmon resonance.

The results obtained with the 8-nt sequence of the aptamer loop, 5’-GUCCAGA-3’, the six central bases of which are complementary to the TAR RNA loop, compared with those obtained with the R-0624(GA) aptamer clearly suggest a role for the stem in loop-loop complexes. It supports a view where the higher stability of the complex with the aptamer over the one with the antisense sequence (ΔT<sub>m</sub> = +20 °C) results from the interaction of two structured motifs in agreement with previous results (36). NMR studies on the complex formed between HIV-1 TAR and TAR*, the rationally designed hairpin RNA with a fully complementary loop, have shown that there is a continuous stacking from the 3'-side of one stem helix through the loop-loop helix to the other stem helix (37). Similar stacking was observed in the loop-loop interaction between RNA I and RNA II, the two RNA hairpins involved in the regulation of the ColE1 plasmid replication (38). The three-dimensional structure of TAR-R-0624 complex is not known yet, but one can reasonably expect a similar structure as indicated by enzymatic footprints and by the fact that loop mutants of R-0624 or R-0616(UA) RNA have the same base composition. They only differ in the stem sequence below the two first base pairs next to the loop: GCC-CGA for the former and CAC-GUG for the latter. Influence of base pairs, next to the loop, on kissing complex stability has been reported in the RNA I-RNA II loop-loop interaction (6). As observed for this complex, the *in vitro* selection against TAR RNA identified 5’-purine-pyrimidine and 5’-pyrimidine-purine base pair preference at the first and second positions of the aptamer stem, next to the loop, respectively, which might indicate that this could be crucial for stability. The results obtained with the truncated R-0624(UA) aptamer variant suggest that sequence variations further down the aptamer stem has to be considered too. As for TAR*, the rate constants with CA or CU variants could not be determined, but in this case only the loop closing pair was modified. Thus, both the sequence of the stem close to the loop and the closing pair can be kinetically critical for fast interaction in the time scale of the SPR experiments.

The stability of TAR-aptamer complexes decreases in the order GA > AG > GG > GU > AA > GC > UA >> CA, CU. Roughly the same ranking was observed for hexanucleotide hairpin loops (39) and internal mismatches in RNA (40). Purine stacking interactions at the loop-loop helix/stem junction have been reported for the complex between the two RNA transcripts of the ColE1 plasmid (38). Such interactions might also stabilize the TAR RNA-aptamer complex as, except for the GU aptamer mutant, the PuPu mutations are the less destabilizing ones. Interestingly, the stability of the complexes analyzed with SPR and with UV-absorption spectroscopy follows the one found with EMSA experiments (25). Thermal denaturation experiments monitor the equilibrium in solution, whereas SPR (one of the partners is immobilized on a surface) or EMSA (the molecules migrate in a three-dimensional network) do not. Despite these fundamental technical differences, the results demonstrate that the three techniques are following the same molecular event. Even if the absolute values of the equilibrium binding constant for the complex with the aptamer or with aptamer does not give rise to the strongest TAR binder (ΔT<sub>m</sub> = −15.9 °C compared with the wild-type complex). The stabilizing role of the GA pair is further emphasized by the comparison with TAR*. This hairpin RNA has a stem that is 3 base pairs shorter than the aptamer, but it is actually equivalent to the R-0624(UA) variant in terms of loop complementary and closing pair. Identical T<sub>m</sub> values (about 30 °C) for TAR-TAR* and TAR-R-0624(UA) complexes illustrate this point (Table I). Furthermore, a truncated version of R-0624(UA), R-0614(UA), now also equivalent to TAR* in terms of stem length (5 base pairs), gives a T<sub>m</sub> for the bimolecular complex equal to about 30 °C too (Table I). This indicates that despite the lower intrinsic stability of this truncated mutant (T<sub>m</sub> = 62.9 °C) compared with the full-length mutant (T<sub>m</sub> = 75.3 °C), the higher affinity of R-0624(GA) aptamer (K<sub>d</sub> = 2.0 nM) over the rationally designed TAR* RNA (K<sub>d</sub> = 92.5 nM) originates essentially in the GA pair.

Kinetics analysis of mutants indicates that the equilibrium binding constant is controlled by the off-rate rather than by the on-rate. Clearly, the GA pair stabilizes the complex between the aptamer and TAR RNA once formed and prevents it from faster dissociation as observed with the aptamer variants. Under the buffer conditions of the *in vitro* selection (3 mM magnesium), binding of TAR* to the immobilized TAR RNA is so poor that the kinetics could not be determined. However, we could determine rate constants with the aptamer mutant equivalent to TAR* in terms of loop and closing pair, R-0624(UA), and even with its truncated version, the TAR* like R-0614(UA) (data not shown). Then, TAR* and the UA variants are not kinetically equivalent, TAR* and the truncated R-0614(UA) RNA have the same base composition. They only differ in the stem sequence below the two first base pairs next to the loop: GCU-CGA for the former and CAC-GUG for the latter. Influence of base pairs, next to the loop, on kissing complex stability has been reported in the RNA I-RNA II loop-loop interaction (6). As observed for this complex, the *in vitro* selection against TAR RNA identified 5’-purine-pyrimidine and 5’-pyrimidine-purine base pair preference at the first and second positions of the aptamer stem, next to the loop, respectively, which might indicate that this could be crucial for stability. The results obtained with the truncated R-0614(UA) aptamer variant suggest that sequence variations further down the aptamer stem has to be considered too. As for TAR*, the rate constants with CA or CU mutants could not be determined, but in this case only the loop closing pair was modified. Thus, both the sequence of the stem close to the loop and the closing pair can be kinetically critical for fast interaction in the time scale of the SPR experiments.
TAR* show a discrepancy, the linear correlation between the binding equilibrium constant determined by SPR and the $T_m$ (Fig. 8) validates non-equilibrium techniques and supports our conclusions.

Specific binding of monovalent and particularly divalent cations such as magnesium ions to RNAs is often required to proceed with the biological processes in which these RNAs are involved (41). These cations are trapped locally and interact either directly or through water molecules (42, 43). The stabilizing role of magnesium ion on RNA kissing complexes is known (5, 6). Strong evidence of direct binding of this cation to the dimerization initiation site of HIV-1 has been reported recently (8). One magnesium would bind at the center of the pocket in the sharp turn that each loop makes. Similar binding was proposed for the ColE1 plasmid (38). The analysis of the three-dimensional structure of TAR-TAR* complex suggests that the two phosphate clusters flanking the major groove of the loop-loop interaction helix may constitute part of two specific metal ion binding sites (37). The number of magnesium ions that bind upon formation of TAR-TAR*, deduced from the ion concentration dependence of the $T_m$ agrees well with this hypothesis. In vitro selection identified high affinity RNA ligands for TAR RNA at physiological magnesium concentration (3 mm). According to our results, a similar number of magnesium ions seem to be directly implicated in the formation of the complex with the aptamer. Then the higher affinity of the aptamer ($K_a = 2.0 \text{ nM}$) for TAR RNA over the rationally designed TAR* binder ($K_a = 92.5 \text{ nM}$) does not reside in a difference in the number of magnesium ions that bind. Supported by the results obtained with the aptamer variants, we hypothesize that the loop closing pair of the R-0624(GA) aptamer is critical for the stability of the complex with TAR RNA. Whether the GA pair is a noncanonical pair is not established. The data cannot give direct evidence of that. Clearly the GA pair is a structural determinant, which is fundamental to explain the higher stability of the TAR RNA-aptamer complex at physiological concentration of magnesium ion over any other one, including the complex with the rationally designed TAR* ligand. The role that the aptamer stem might also play is presently under investigation. This validates the usefulness of an in vitro combinatorial approach over a rational one to identify high affinity RNA ligands. The GA pair favors the stability of the complex once formed as the binding equilibrium constant is controlled by the off-rate rather than by the on-rate of the complex formation. Finally, GA pair could be preferred when structural distortions that might increase stability of loop-loop RNA complexes are required.

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Note Added in Proof—Recently, a report of the interaction of a truncated TAR RNA with TAR* was published (Nair, T. M., Myszka, D. G., and Davis, D. R. (2000) Nucleic Acids Res. 28, 1955–1940). The binding equilibrium constant determined by surface plasmon resonance agrees well with our results.

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FIG. 8. Correlation between the equilibrium binding constant, $K_a$, and $T_m$. $K_a$ for the complexes between miniTAR and the aptamers, in solution, for the complexes between miniTAR and the aptamers.

Closing GA Pair in Loop-Loop RNA Complexes

that the loop closing GA pair is not a sheared pair.

Could a closing GA pair be preferred to increase loop-loop complex stability? Recently, an in vitro selection against yeast tRNA$^{\text{phe}}$ identified an aptamer that folds as a hairpin (46). The seven central bases of the 9-nt loop of this RNA are complementary to the entire tRNA$^{\text{phe}}$ anticodon loop and are also closed by a GA pair. Together with our results, this work suggests that, when all positions are randomized, a GA pair is preferred to close a loop. Thus, this extra pair would be thermodynamically favorable for loop-loop interactions. To check this hypothesis, the Watson-Crick GC pair of RNA $I'$, next to the loop, was replaced by a GA pair. Despite the lower stability that this new hairpin displays ($\Delta T_m = -5.8 \degree C$), the resulting loop-loop complex with RNA II' shows a clear transition at about 24 $\degree C$, which is not observed with the unmodified RNA I' hairpin. The extra-GA pair is clearly favorable even with the stem shortened by 1 base pair that results from this mutation.

In conclusion we showed that the GA pair that closes the loop of the in vitro unidentified R-0624(GA) aptamer is critical for the stability of the complex with TAR RNA. Whether the GA pair is a noncanonical pair is not established. The data cannot give direct evidence of that. Clearly the GA pair is a structural determinant, which is fundamental to explain the higher stability of the TAR RNA-aptamer complex at physiological concentration of magnesium ion over any other one, including the complex with the rationally designed TAR* ligand. The role that the aptamer stem might also play is presently under investigation. This validates the usefulness of an in vitro combinatorial approach over a rational one to identify high affinity RNA ligands. The GA pair favors the stability of the complex once formed as the binding equilibrium constant is controlled by the off-rate rather than by the on-rate of the complex formation. Finally, GA pair could be preferred when structural distortions that might increase stability of loop-loop RNA complexes are required.

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