Evidence for tertiary structure in natural single stranded RNAs in solution

Sylvie Ghribi1, Marie-Christine Maurel2, Michel Rougec3 and Alain Favre*

Institut J. Monod, Laboratoire de Photobiologie Moléculaire, 2 Place Jussieu, 75251 Paris Cédex 05, France

Received November 20, 1987; Accepted January 13, 1988

ABSTRACT:

Binding isotherms (20°C) of ethidium bromide to a number of tRNA species at various ionic strengths indicate that i) the number nI of Intercalation sites is high 7 to 11 per molecule, in the low salt form III, but small, 2 to 1, at high Mg2+ or Na+ when form I predominates. ii) modification of tRNA at strategic positions for 3D folding prevents full expression of Intercalation restriction iii) maximal restriction is obtained at salt concentrations higher than needed for full conversion to form I. It is inferred that restriction, which is not observed with bilhelical RNA (or DNA), requires the native tRNA 3D structure but also some physical coupling between the region of 3D folding and bilhelical arms.

Ribosomal RNAs, some viral RNAs, mRNA from sheep mammary gland as well as the random copolymers Poly UG, Poly AUG, Poly AUCG all exhibit Intercalation restriction. Hence 3D folding of the polyribonucleotide chains appears to be a feature common to single-stranded RNAs when free in solution under physiological conditions.

INTRODUCTION

Cellular RNAs that play a central role in the expression of cellular genetic information as well as most viral or viroid RNAs are single-stranded molecules. Our knowledge of the conformation of these RNAs when free in solution or when embedded in ribonucleic particles is still limited with the exception of the few tRNA species analysed at atomic resolution by X-ray crystallography (1). Evidence for the presence of secondary structure (double-stranded perfect or imperfect mini-helices connected by loops) was obtained some years ago for ribosomal RNAs, eucaryotic messengers and viral RNAs (see ref. in 2). Given an RNA primary sequence it is now relatively easy to obtain the corresponding more stable secondary structures. In general several models can be proposed and phylogenetic data when available provide a decisive clue to select the more relevant 2D structure (and variants) as illustrated in the case of rRNAs (3). Also RNA-RNA crosslinking experiments have demonstrated the tertiary folding of rRNAs inside the ribosome (4). On the other hand direct physico-chemical evidence for a 3D structure of messenger or viral RNAs is still lacking. This situation constrasts with the recent discovery of ribozymes i.e. RNA molecules exhibiting catalytic activity. Hence the RNA component of RNase P is able to catalyse the specific hydrolysis of tRNA precursors (5). Autoexcision of the IVs intron
Nucleic Acids Research

of Tetrahymena RNA occurs in the absence of any energy source (6). It seems obvious that some kind of tertiary structure is implied in these ribozymes activities.

Our purpose here was therefore to develop a simple and sensitive assay allowing the detection of single-stranded RNA tertiary structure. We have previously reported the use of the dye ethidium bromide, EB, which becomes highly fluorescent when intercalated into the double-stranded regions of nucleic acids (7) to detect RNA secondary structure (8). The new developments presented here are essentially based on the observation that native tRNAs have much less intercalation sites, \( n_i = 1 \) to 2, than expected on the basis of their secondary structure and of the known intercalation properties of the dye into double-stranded polynucleotides (9-10). In the first part of the present work we have examined the relationship between \( n_i \) and the tRNA 3D structure followed by means of spectroscopic and photochemical probes. This led us to establish that the presence of the native 3D structure is a prerequisite to intercalation restriction. We have then applied this fluorimetric procedure to a variety of polynucleotides including ribosomal and viral RNAs as well as messenger RNA and random copolynucleotides. These single-stranded RNAs were found to exhibit intercalation restriction in contrast to bihelical RNA.

MATERIAL AND METHODS.

a) Ethidium bromide.

3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (Sigma) was dissolved in bidistilled water and insoluble particles removed by low speed centrifugation. Stock solutions (10mM) were kept at 4°C in the dark. Before each experiment the visible absorption spectra was recorded. An intact sample has an absorption ratio at 480nm to 380nm of 10. Concentration was determined taking \( \epsilon_{480} = 5600 \, \text{M}^{-1}\cdot\text{cm}^{-1} \) (9).

b) Polynucleotides.

Bulk tRNA as well as tRNA\(^\text{Val}\), tRNA\(^\text{Phe}\), tRNA\(^\text{Met}\), tRNA\(^\text{Lys}\), tRNA\(^\text{Tyr}\), tRNA\(^\text{Glu}\) from E. coli MRE 600, tRNA\(^\text{Phe}\) from yeast and MS\(_2\) RNA were from Boehringer Manheim. Some bulk tRNA samples as well as the 5S, 16S and 23S were prepared by standard procedures. mRNA from sheep mammary gland is a mixed population of mRNA molecules obtained from the non induced gland as described (11). It was kindly given to us by Dr L. Houdebine (CNRZ Jouy en Josas). TMV and TYMV viral particles were sent to us by Dr H. Guilley (IBMC Strasbourg) and viral RNA was prepared by phenol extraction. Random polynucleotides PolyUG, PolyAUG, PolyAUCG synthesized with polynucleotide phosphorylase were obtained from Choay Chimie. Proportions of the different nucleotides were close to 1 in these polymers. Double-stranded viral RNA was
prepared from the virus like particles (VLP) found associated with a number of yeast strains. VLP from strain CBS 6 124-2 of Yarrowia Lipolytica was kindly given to us by Dr Treton (Laboratoire de Génétique de l'RNA Paris). After phenol extraction the double-stranded RNA was purified from single-stranded contaminating RNA by chromatography on CF11 Whatman cellulose as described (12). Concentrations were determined by absorption at 260nm in a renaturing buffer containing sodium cacodylate 5mM pH 7, NaCl 0.1M and MgCl2 10 mM using a molar extinction coefficient $\varepsilon_{260}$ of 7 400 M$^{-1}$.cm$^{-1}$ per nucleotide for single stranded RNAs and of 6 800 M$^{-1}$.cm$^{-1}$ for double-stranded RNA. 1 A$^{260}$ unit is the quantity of RNA yielding an absorbance of 1 at 260nm in a 1cm path length cuvette when dissolved in 1 cm$^3$ of the above buffer. The reference buffers used here are sodium cacodylate 0.1 mM pH 7 without MgCl2 (buffer A) and plus 10 mM MgCl2 (buffer B).

c) Divalent cations removal.

The RNA samples (2 to 40 A$^{260}$ units) dissolved in bidistilled water were successively dialysed in the dark at 4°C against: a) NaCl 0.1M, EDTA 20mM, sodium cacodylate 0.1mM pH 7 b) NaCl 0.1M, sodium cacodylate 0.1mM pH 7 c) buffer A.

Each step of dialysis was for at least 12 hours. It was checked by atomic absorption that after this treatment, tRNA samples contains less than 0.5 Mg$^{2+}$ per molecule. Concentrated samples were either used immediately or divided into aliquots of 5 A$^{260}$ units and kept at -20°C until use. Purity and integrity of the RNAs after chemical treatment, or divalent cations removal (see below) were checked by gel electrophoresis either on 10% polyacrylamide, urea 7M gels (13) for the tRNAs and 5S RNA or on 1.5% agarose gels containing formaldehyde 2,2 M for 16S, 23S rRNAs and the viral RNAs (14). After migration RNA bands were stained with ethidium bromide and revealed using the dye fluorescence.

d) Spectroscopic techniques.

Absorption spectra were measured with a Cary 15 spectrophotometer. Phosphorescence and fluorescence measurements were performed with a Jobin Yvon spectrofluorometer using thermostated (20°C) 600 μl microcuvets. For phosphorescence measurements ($\lambda$ exc 340nm, $\lambda$ em 510nm) band-passes of 30nm were used. For detection of the reduced 8-13 link ($\lambda$ exc 390nm ; $\lambda$ em 450nm) the band-passes were 10 nm (see below).

e) tRNA crosslinking.

Illumination of the tRNA samples was performed with a Lantern Cunow System equipped with an HBO 200 W superpressure mercury lamp and a MTO J 310 filter. Four or five cuvets placed 5cm from the exit slit of the lantern on a 20°C thermostated holder were simultaneously
Irradiated. Kinetics of 8-13 link formation were determined with bulk tRNA, 5A260 units in 1ml of the appropriate pH 7 buffer. 100 µl aliquots were taken at appropriate times during irradiation and processed as described below. The extent of the 8-13 link formation was determined on samples irradiated for at least 45 min i.e. six times the half-time of the reaction. The NaBH₄ reduction treatment (15) was applied on samples containing 0.5 A₂₅₀ unit of tRNA. All irradiated samples in a series were first adjusted to the same final salt concentration i.e. 50 mM sodium cacodylate pH 7, 10mM MgCl₂ and when necessary 0.4 M NaCl. A 0.9 M NaBH₄ solution was then adjusted to pH 9.7 by addition of 1 M NH₄OH and 100 µl of this mixture was added to the tRNA samples. Reduction was performed either overnight at 4°C or at room temperature for 5 hours. 100 µl of 1M sodium acetate pH 4.5 were then added allowing neutralisation of the solution and degradation of the NaBH₄ excess. Fluorescence of the reduced 8-13 link was then measured.

f) Ethidium bromide intercalation in nucleic acids.

The RNA sample, with an initial volume of 600 µl, and initial concentration in general of 70 µM nucleotides, was titrated at 20°C by the sequential addition of dye (stock solutions 0.1 and 1mM). After addition of the dye the solution was left to equilibrate for a few minutes and the fluorescence signal I₁ (λexc 540nm - λem 600nm) was then measured. When necessary I₁ was corrected for eventual (weak) RNA sample diffusion. I₁ is related to c the concentration of added dye by I₁ = k (c - cᵢ) + k V cᵢ where k is a constant, V the fluorescence stimulation factor and cᵢ the concentration of intercalated dye. When the same titration experiment was performed in the absence of RNA one obtains: I₀ = k c, c ≤ 0.03 mM, allowing the determination of the constant k by linear regression. cᵢ is given by:

\[ cᵢ = (I₁ - I₀) / k (V-1) \quad \text{and} \quad cᵢ = c - cᵢ = (I₀ V - I₁) / k(V-1). \]

The V parameter was determined from the ratio of the fluorescence signal I₁ of a dye solution in the presence of a saturating amount of tRNA to I₀, the corresponding signal of the free dye at the same concentration. The r = cᵢ/cRNA and r/cᵢ parameters were calculated from experimental values on a Data General Eclipse S/130 computer using a program kindly provided by Dr F. Rodier and plotted according to Scatchard (16). Fluorescence decay curves (exc 330nm, em 600nm) were obtained in the conditions used for titration experiments with a single-photon counting apparatus (Edinburgh Instruments).
RESULTS AND DISCUSSION

Dyes such as acriflavine (17), proflavine (18) or ethidium bromide (9, 10) intercalate in tRNA with high affinity, (K_D in the micromolar range), but can also bind by electrostatic interactions to the negatively charged phosphate groups. Large variations in the evaluation of n_i the number of intercalation sites have been found, from 1 to 10 per tRNA molecule, depending on the drug and the tRNA species under examination and on the conditions used. In two of these studies (9, 17) it was clearly established that folding the tRNA chain in a rigid 3D structure appreciably restricts intercalation. tRNA can be found under four phases or forms (19) and our first aim was therefore to examine their relationship with n_i. Of peculiar interest here are forms I and III that can be obtained at room temperature. Form I predominates at high ionic strength and corresponds closely to the native L-shaped tRNA structure (20). Form
Fig 2 The Na⁺ (expressed in moles/l) induced form III to form I transition in bulk E. coli tRNA at 20°C.

a) addition of Na₂SO₄, C_{RNA} = 4 mM : absorption at 340nm -Δ-Δ-Δ-, phosphorescence emission at 510nm -•-•-•-.  

b) addition of Na₂SO₄ : n₁ -•-•-•- C_{RNA} = 70 µM ; reduced 8-13 link fluorescence F_{450} -○-○-○-○- C_{RNA} = 670µM. The dotted line -•-•- represents the transition assuming fast equilibrium.  

c) addition of NaCl : n₁ -•-•-•- C_{RNA} = 70 µM ; F_{450} -□-□-□- and K the apparent rate constant for 8-13 link formation -▲-▲-▲- C_{RNA} = 670 µM.

III is found in the absence of divalent cations under low monovalent salt conditions (19). This form has lost the native 3D folding but kept extensive secondary structure and we have recently shown that form III tRNA^{Phe} is a complex mixture of conformers (21).
TABLE I
Number of intercalation sites and mid-transition parameters for tRNAs.

<table>
<thead>
<tr>
<th>tRNA</th>
<th>$n_i$</th>
<th>$\text{Mg}^{2+}$ at mid-transition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>form I</td>
<td>form III</td>
</tr>
<tr>
<td>Total</td>
<td>1.5</td>
<td>5.2</td>
</tr>
<tr>
<td>Val 1</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Met f</td>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Phe</td>
<td>2.0</td>
<td>5.2</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.0</td>
<td>5.2</td>
</tr>
<tr>
<td>Phe (yeast)</td>
<td>1.0</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Form I is obtained by renaturation of form III by addition of $\text{Mg}^{2+}$ to a final concentration of 10 mM. $n_i$ is the number of intercalation sites per tRNA molecule. The concentration of $\text{Mg}^{2+}$ at the mid-transition $\text{Mg}_T^{2+}$ (expressed in $\mu$M) have been scaled for a tRNA concentration (expressed in nucleotides) of 70 $\mu$M, the concentration used in the EB titration experiments.

a) Determination of $n_i$ in high and low salt.

The $\text{Mg}^{2+}$ free RNA samples in buffer A (see Methods) were adjusted to a defined $\text{Na}^+$ (or $\text{Mg}^{2+}$) concentration by addition of NaCl, $\text{Na}_2\text{SO}_4$ (or $\text{MgCl}_2$). The solutions generally 70 $\mu$M in nucleotides were left to equilibrate at 20°C for at least 20 min before starting the fluorimetric titration with ethidium bromide. The $k$ and $V$ parameters were determined as described in Methods. Unlike $k$, $V$ was found to vary with the ionic concentration (insert of Fig. 1) and this was taken into account. The Scatchard plots obtained with bulk tRNA indicate that more binding sites are available at 0.1 mM $\text{Na}^+$, $n_i \sim 5$ (Fig 1) than in 1M NaCl, $n_i \sim 1.4$ (Fig 2) or 10 mM $\text{Mg}^{2+}$ $n_i \sim 1.5$. The same conclusion applies to a number of tRNA species (Table 1). In the analysis above $V$ is assumed to be constant whatever the extent of binding. This is obviously not the case in low salt. As the total dye concentration increases (Fig 1) $r$ reaches a maximal value close to 5 and then decreases. This phenomenon is due to energy transfer from
Intercalated EB to outside electrostatically weakly fluorescent bound dyes resulting in fluorescence quenching (7).

To evaluate the variations of $V$ the EB fluorescence decay curves were determined with bulk tRNA using a single photon counting apparatus. Conditions were those of the titration experiments. In high salt i.e. 1M Na$^+$ or 10 mM Mg$^{2+}$ a biexponential decay is obtained with $\tau_i = 1.6$ nsec accounting for free EB. The lifetime $\tau_i$ corresponding to intercalated EB remains stable at $25 \pm 0.5$ nsec until $r$ reaches 0.5 and then decreases smoothly $\tau_i = 24 \pm 0.5$ nsec when $r$ approaches 1. This variation reflects heterogeneity of Intercalation sites and possibly some quenching. Since all bound dyes have very similar absorption characteristics (7), the $V/\tau$ ratio should to a first approximation remain constant leading to the conclusion that the equilibrium titration procedure underestimates $n_i$ by at most 15%. In 0.1 mM Na$^+$ three exponential terms are required at apparent $r$ values higher than 1 to fit the experimental fluorescence decay curve. Of the three corresponding lifetimes the shortest one $\tau_1$ corresponds to free dye. The two $\tau_i$ decrease respectively from 25 to 22 nsec and from 15 to 11 nsec when $r$ increases from 1 to 4.5. At a dye tRNA ratio of 31 (mole/mole) leading to decreasing $r$ values, $r = 4.02$, the $\tau_i$ become 12 and 7 nsec. Clearly quenching becomes very efficient under these conditions and the need for two $\tau_i$ merely reflects the heterogeneity of emitting, intercalated dyes with respect to quenching. An analysis of these data not detailed here shows that: i) a significant fraction of the added dye, 20%, is already bound electrostatically at $r$ values close to 1; ii) the actual number of sites able to stimulate the EB fluorescence is in the range of 7 to 11 per tRNA molecule. Hence the titration procedure yields realistic $n_i$ values in high salt but largely underestimates $n_i$ in low salt and this apply equally well to other RNA samples as shown by a similar investigation of EB binding to rRNA.

In conclusion low and high salt tRNAs exhibit widely different number of intercalation sites. Low salt tRNA$^{Phe}$ is known to be a mixture of conformers (21) and Intercalation is expected to preferentially stabilize some of them. On the other hand the conformational perturbation induced by EB intercalation should be quite limited in high salt since $n_i \leq 2$. Hence $n_i$ might not represent the structural features of the original molecule (or form) but rather reflects ligand induced conformational change. In order to closely analyse the relationship between $n_i$ and the tRNA forms $n_i$ was determined all along the form III to form I transition.

b) Na$^+$ induced form III to form I transition.

The transition was followed by means of the built-in probe 4-thiouridine found almost
Fig. 3 The Mg$^{2+}$ (expressed in moles/l) induced transition between form III and form I in bulk E. coli tRNA, $C_{\text{tRNA}} = 4$ mM: absorption at 340nm - - - - - , phosphorescence at 510nm - - - - - , $F_{450}$ - - - - - .

exclusively at position 8 of 70% of E. coli tRNA molecules. Position 8 is always occupied by a uridine (or its 4-thiouridine analog) and is found base-paired in the native tRNA structure with another universally present residue A14 (22). In bulk tRNA we have used the specific absorption of 4-thiouridine at 340nm, $A_{340}$, its room temperature phosphorescence $P_{510}$ and the photochemical formation of the 8-13 link, Pyo(4-5) Cyt, measured by the fluorescence $F_{450}$ of NaBH$_4$ reduced irradiated tRNA (15). The 8-13 link adduct is formed quantitatively in all tRNA species containing the 4-thiouridine 8 and cytidine 13 residues in conditions favouring form I tRNA (23). On the other hand cytidine 13 is not involved in the weak amount of photoadduct that can be obtained in form III tRNA$^{\text{Phe}}$ (21). Hence the $F_{450}$ values determined at the plateau of the photoreaction ($F_{450} = 1$ in form I tRNA) are directly related to the % 8-13 link formed (Fig 2 and 3). The kinetics of Pyo(4-5) Cyt formation remains roughly first-order whatever the Na$^+$ concentration. With Na$_2$SO$_4$ the apparent rate constant $k$ varies in parallel with the $F_{450}$ and $P_{510}$ signals. The same behaviour is obtained with NaCl until the Na$^+$
concentration reaches 0.2 M. Above this threshold concentration $F_{450}$ and $k$ decreases in parallel (Fig 2c) due to quenching of the 4-thiouridine triplet state by Cl$^-$ (25).

Since the rate constant $k$ varies, $F_{450}$ is a direct measure of $f_1$ the fraction of molecules under form I at time 0 of the photoreaction only if form I and form III do not interconvert in the time-scale of the photoreaction. As discussed in Appendix this does not appear to be the case. If form I and form III are in fast equilibrium the mid-transition point $f_1 = f_2 = 0.50$ will correspond to $F_{450} = 0.740$. As a consequence the transition between the two forms is steeper than shown by the $F_{450}$ variations and the mid-transition Na$^+$ concentration is shifted from 0.03 to 0.05 Na$^+$. Within the range of tRNA concentrations used, the free Na$^+$ concentration is in close approximation equal to total Na$^+$ and the data can be compared as shown in Fig. 2. Clearly the $A_{340}$, $P_{510}$ and $F_{450}$ signals although differently weighted in total tRNA since 25% of 4-thiouridine containing tRNAs, such as tRNA$^{Ser}$, tRNA$^{Tyr}$ or tRNA$^{Giu}$, lack the cytidine in position 13 and are not crosslinkable (24), exhibit closely related variations. This indicates that the transition occurs concomitantly in the various tRNA species a finding supported by the closely similar $F_{450}$ data obtained with tRNA$^{Val}$ (data not shown).

The number of intercalation sites as a function of the Na$^+$ concentration was determined by the fluorimetric titration procedure (Fig 1 and 2). Taking into account quenching effects increase the amplitude of intercalation restriction but leave unaffected the shape of the curve. Hence the variation of $n_I$ accompanies the conformational transition with however a larger breadth and higher mid-transition Na$^+$ concentration for both bulk tRNA (Fig 2b and 2c) and tRNA$^\text{1Val}$. Particularly striking is the fact that full conversion to form I is achieved at 0.2 M Na$^+$ although maximal intercalation restriction occurs when the Na$^+$ concentration approaches 1M.

c) Mg$^{2+}$ induced form III to form I transition.

Experiments were conducted as described above for Na$^+$ renaturation and extended to a number of different tRNA species. For bulk tRNA the variations of $A_{340}$, $P_{510}$ and $F_{450}$ are closely related when performed on tRNA samples at the same concentration (Fig. 3). Amplitude of the $P_{510}$ stimulation in bulk tRNA as well as tRNA$^\text{1Val}$ and tRNA$^\text{Phe}$ (not shown) is larger than obtained with Na$^+$. The rate of 8-13 link formation increases again two times upon tRNA renaturation as shown previously with Na$^+$ and quenching again occurs at high Cl$^-$ concentrations. In the low monovalent salt conditions used, the total concentration of Mg$^{2+}$ at the mid-transition, Mg$^+_1^{2+}$, becomes highly tRNA concentration dependant (Fig 3 & 4). For
The Mg\textsuperscript{2+} (moles/l) induced form III to form I transition in *E. coli* tRNA\textsuperscript{Phe} as followed by the variation of \( n_i \) (per tRNA molecule) - - - - - - - C\textsubscript{RNA} = 70 \mu M, or F\textsubscript{450}

\[
\text{C}_{\text{RNA}} = 670 \mu \text{M} \quad \square \square \square \square \square \text{, C}_{\text{RNA}} = 70 \mu \text{M} - - - - - - -.
\]

bulk tRNA studied at three concentrations \( \text{C}_{\text{RNA}} = 4 \text{ mM, 0.67 mM and 70 } \mu \text{M} \) respectively one finds \( \text{Mg}^{2+} \approx 0.16 \pm 0.1 \text{ C}_{\text{RNA}} \) i.e. it needs about 12 bound Mg\textsuperscript{2+} cations to renature 50% of the molecules. This compares satisfactorily to previous data obtained with tRNA\textsuperscript{Met} in 10 mM Na\textsuperscript{+} (26). Hence in the following Mg\textsuperscript{2+} could only be compared in experiments scaled at the same RNA concentration.

All tRNA species, including tRNA\textsuperscript{Tyr} from *E. coli* and tRNA\textsuperscript{Phe} from yeast exhibit intercalation restriction (Table 1). In general when the transition is achieved there remains only one strong binding site per molecule. At 10 mM Mg\textsuperscript{2+} the corresponding binding constants are in the range of 1 to 10 \( \mu \text{M}^{-1} \) and decrease in the following order for *E. coli* tRNAs: tRNA\textsuperscript{Val}, bulk tRNA, tRNA\textsuperscript{Phe}, tRNA\textsuperscript{Met}. The strong binding site for tRNA\textsuperscript{Phe} yeast was proposed to be localized between base pairs AU\textsubscript{6} and AU\textsubscript{7} of the CCA arm on the basis of NMR (27) and energy transfer experiments (10). This figure is consistent with several additional observations. tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Met} from *E. coli* have G-C rich CCA arms and we have observed that in contrast to other standard RNA homoduplexes, the Poly G . Poly C complex does not bind EB. In addition introduction of the 8-13 link in a variety of *E. coli* tRNA species systematically...
TABLE II
Intercalation sites after NaBH₄ treatment of tRNAs.

<table>
<thead>
<tr>
<th>susceptible bases</th>
<th>bulk</th>
<th>Val</th>
<th>Imit</th>
<th>Tyr</th>
<th>Lys</th>
<th>Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td>s⁴U</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8, 9</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>m⁷G</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td></td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>17</td>
<td>20, 47</td>
<td></td>
<td>16, 17, 20</td>
<td>0</td>
</tr>
<tr>
<td>Ac⁴C</td>
<td>+</td>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>n₁ without reduction</td>
<td>1.5</td>
<td>1.5</td>
<td>1</td>
<td>1</td>
<td>1.5</td>
<td>1.6</td>
</tr>
<tr>
<td>n₁ with reduction</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3.8</td>
<td>4.5</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Mild NaBH₄ reduction of tRNAs in buffer A was performed as described in Results. EB titration was carried out in buffer B. The table indicates the localisation of NaBH₄ susceptible residues in the tRNA sequences as well as the experimental value of n₁ (per tRNA molecule). In bulk tRNA, 4-thiouridine, s⁴U₈, and 7-methyl-guanosine, m⁷G₄₆, are present in 70% of the molecules. Dihydouridine, D, and N⁴-acetyl-cytidine Ac⁴C are only found in specific tRNA species (40).

increases the binding constant of the strong site by a factor of 2 to 3 (A. F. unpublished results). Except for NMR studies the data above are also consistent with a close but distinct localisation found after diffusion of EB in tRNA crystals. In both yeast tRNA⁰Phe (28) and yeast tRNA⁰Asp crystals (E. Westhof private communication), the dye is found bound inside the corner of the L shaped structure. One possibility is that constraints in crystalline tRNA prevent intercalation at the end of the CCA stem but allows EB to fix at a closely related "preintercalation site". Determination of the fluorescence yield of the dye in the crystal will help to solve this question.

Taking into account the tRNA concentration, C₉RNA = 70μM, used in dye titration experiments makes it clear that intercalation restriction again accompanies the form III to form I transition (fig. 4). Full restriction occurs at Mg²⁺ concentrations much higher than needed for conversion to form I. This is certainly due in part to the fact that EB which has more binding sites in form III will preferentially stabilize it and accordingly displace the transition to higher Mg²⁺ concentration. Alternatively the possibility exists that at the lowest salt concentration allowing form I to predominate, intercalation is still allowed in bihelical regions and particularly those (CCA and anticodon arms) not involved in tertiary interactions. Increasing the Mg²⁺ concentration is well known to increase the coupling between the different tRNA regions as
judged for example by the cooperative character of thermal melting in high salt (9, 19). The
bihelical arms are then fixed on the double helix A form found in crystals (1) and their limited
internal mobility will prevent intercalation. In agreement with this view is the fact that a single
base bulge in small hairpin helices is able to induce structural effects at distance (29). The
Mg\textsuperscript{2+} cation which exhibits strong and weak binding sites to tRNA (30) could also prevent
intercalation by competitive binding. It should be noticed however that the same limiting values
of n\textsubscript{i} are found in high monovalent salt (Fig 2). In summary our data show that the native 3D
folding of tRNA, when tight, induces intercalation restriction. They do not exclude the possibility
that "wrong" folding could behave as well.

d) Intercalation in NaBH\textsubscript{4} reduced tRNAs.

Preliminary observations have shown that extensive reduction of bulk tRNA (3 hours
with 2M NaBH\textsubscript{4}) in the presence of 10 mM Mg\textsuperscript{2+} abolishes the tRNA amino-acid acceptor
 capacities and increases the number of intercalation sites. Ribose-phosphate cuts can be detected
however by gel electrophoresis. A milder procedure close to the one used by Wintermeyer and
Zachau (31) was therefore used for purified tRNA species. Mg\textsuperscript{2+} free tRNA samples in buffer A
were brought to pH 9.8 by NH\textsubscript{4}OH addition. Reduction was performed for three hours in the dark
with 3 mM NaBH\textsubscript{4}. As shown in Table 2 all tRNA species containing NaBH\textsubscript{4} susceptible bases
exhibit an increased number of intercalation sites when titrated in conditions where form I tRNA
normally prevails. On the other hand the same treatment slightly increases the dye binding
affinity but does not notably affect n\textsubscript{i} when applied to tRNA\textsuperscript{Glu} which lacks any such bases. In
tRNA crystals (22) uridine 8 and 7-methylguanosine 46 are involved in tertiary interactions
and are found base-paired with adenosine 14 and guanosine 22 respectively. The reduction of
tRNA in high salt presumably disrupts these interactions. When performed on tRNA in low salt
refolding may in addition be impaired.
e) Intercalation into a DNA or RNA duplex.

It is known that the number of intercalation sites in double-stranded DNA or with the
polyribonucleotide duplex Poly A - Poly U is independent upon ionic strength (7). The data were
not extended however to the low monovalent Na\textsuperscript{+} concentration used here and for this reason we
have first examined calf thymus DNA. The DNA was deprived of Mg\textsuperscript{2+} as described above and the
drug DNA interaction studied in buffers A and B. As expected n\textsubscript{i} can be considered invariant n\textsubscript{i} =
0.21 to 0.23 per nucleotide. Natural bihelical RNAs are not easily available but represent
nevertheless the best models we have to study the RNA double helix interaction with the drug.
Double-stranded RNA can be found in non infectious viral like particles, VLP, present in
numerous yeast strains. The VLP from Yarrowia Lypolitica contains a linear double-stranded
Scatchard representation of the binding at 20°C of EB to Yarrowia Lypolitica double-stranded RNA, \( C_{RNA} = 45 \mu M \), a) in buffer A, b) in buffer B.

RNA 1.5 \( \mu \)m long corresponding to 4,900 base pairs (12). 2.5 A\(_{260}\) units from this RNA were prepared. Scatchard plots (Fig. 5) are typical of cooperative fixation at low \( r \) values and \( n_i \) is found constant at low and high salt \( n_i = 0.17 \pm 0.02 \) per nucleotide.

- **Other RNAs.**

    All RNA samples at our disposal (Table III and Fig 6) were freed of Mg\(^{2+}\) by dialysis as described before for tRNAs. Care was taken to preserve the integrity of the molecule which was checked by polyacrylamide or agarose gel electrophoresis. Binding isotherms were established as a function of the Mg\(^{2+}\) concentration. The ribosomal RNAs, 5S, 16S, 23S exhibit a significant amount of intercalation restriction which is roughly 50% with a mid-transition point at Mg\(^{2+}\) concentrations higher than found in tRNA. The presence of 3D structure is expected both on the free RNAs and inside the ribosome since long distance RNA-RNA contacts have been found in 16S RNA (4) and 23S RNA (32). In conditions allowing reconstitution of the 50S particle the 5S RNA can be found under two metastable A & B conformations exhibiting closely similar interactions.
TABLE III
Intercalation restriction in other RNAs.

<table>
<thead>
<tr>
<th>RNA</th>
<th>n_i buffer A</th>
<th>n_i buffer B</th>
<th>Mg^2+ at mid-transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5S</td>
<td>5</td>
<td>2.7</td>
<td>190</td>
</tr>
<tr>
<td>16S</td>
<td>7.5</td>
<td>4.2</td>
<td>110</td>
</tr>
<tr>
<td>23S</td>
<td>8.5</td>
<td>4.5</td>
<td>50</td>
</tr>
<tr>
<td>mRNA</td>
<td>6</td>
<td>2.7</td>
<td>280</td>
</tr>
<tr>
<td>TMV</td>
<td>7</td>
<td>4</td>
<td>200</td>
</tr>
<tr>
<td>TYMV</td>
<td>7.2</td>
<td>4</td>
<td>130</td>
</tr>
<tr>
<td>MS2</td>
<td>7</td>
<td>4.2</td>
<td>300</td>
</tr>
<tr>
<td>Poly AUGC</td>
<td>5</td>
<td>1.7</td>
<td>550</td>
</tr>
<tr>
<td>Poly AUG</td>
<td>2.4</td>
<td>1.3</td>
<td>150</td>
</tr>
<tr>
<td>Poly UG</td>
<td>1.4</td>
<td>0.62</td>
<td>350</td>
</tr>
</tbody>
</table>

n_i is the number of intercalation sites per 100 nucleotides. The total Mg^2+, Mg_{T}^2+ (expressed in µM), at the mid-transition is normalized to C_{RNA} = 70 µM (expressed in nucleotides).

with EB (33). As yet several 3D models have been proposed for the active A form (34).

The second class of RNAs investigated are messenger RNAs. Most of them are of viral origin and carry additional genomic and encapsidation functions. As shown in Table III, intercalation restriction in these RNAs is close to the one found in 16S and 23S RNA. A model indicating extensive 2D structure in MS2 RNA has been proposed and evidence found for tertiary folding (35). The TMV and TYMV plant viruses RNAs are known to exhibit extensive secondary structures. Furthermore at their 3' ends they have acylatable tRNA like structures (respectively by His and Val) and the 3D models proposed for this region mimic to a considerable extent the 3D structure of tRNA (36). In high ionic strength solution both RNAs adopt a compact conformation. Considerably further compaction is needed for TYMV RNA encapsidation. A different process is at work for TMV autoassembly since in the viral particles the RNA is in an open helicoidal form stabilized by coat proteins (37). Probably the most important observation here is the very similar behaviour between mRNAs from non induced mammary gland and the viral and ribosomal RNAs. We also examined a few random copolyribonucleotides. Both Poly AUGC and Poly AUG present evidence for 2D folding as expected (38) but also exhibit a clear-cut
Intercalation restriction (Table III). The data obtained with Poly UG indicate that the U.G. base pair readily participates in the 2D and 3D folding of RNAs.

In conclusion intercalation restriction appears to reflect an intrinsic property of single-stranded RNA chains containing base pairs. It occurs not only in ribosomal RNAs for which evidence for 3D structure already exists but also in viral and messenger RNA. Clearly all these RNAs when free in solution exhibit some kind of 3D folding. It remains to be established whether this tertiary folding persists to some extent when these molecules are involved in biological processes such as replication of viral RNAs or translation of RNA with messenger
functions. The methodology used here would be particularly appropriate to compare the ability of homologous single-stranded DNA and RNA fragments to yield 3D folding.

APPENDIX

Let us call \( f_1 \) (\( f_3 \)) with \( f_1 + f_3 = 1 \) the fraction of tRNA molecules under form I (III) at time 0 of the photoreaction. The \( F_{450} \) signal eventually corrected for the background contribution due to form III tRNA (see Results) is equal to \( f_1 \) only if the two tRNA forms cannot interconvert in the time-scale of the photoreaction. From the data of ref. 39 it can be deduced that at 20°C it takes between 2 sec (tRNA\(^{\text{Ph}}\)) to 2 min (tRNA\(^{\text{Val}}\)) to convert 50% of form III into form I after a salt-jump. Hence, the interconversion rates are fast as compared to the apparent rate constants \( k_1 = 0.2 \pm 0.02 \text{ min}^{-1} \) and \( k_3 = 0.08 \pm 0.02 \text{ min}^{-1} \) for formation of the 8-13 link in form I tRNA and \( s^4U \) photolysis in form III tRNA respectively (see fig 2). Assuming now form III and form I are in fast equilibrium all along the transition led the fraction of unreacted form I tRNA molecules at any time of the photoreaction to remain constant and equal to \( f_1 \). The remaining concentration \( c \) of \( s^4U \) at time \( t \), \( (c = c_0 \text{ at } t = 0) \) is given by:

\[
\text{dc/dt} = k_1 c_1 + k_3 c_3 = k_1 f_1 c + k_3 (1-f_1) c.
\]

\[
\frac{dc}{c} = \left[ k_3 + (k_1-k_3) f_1 \right] \frac{dt}{k_{ap} \cdot dt}
\]

Formation of the 8-13 link is expressed by:

\[
\text{dc/dt} = k_1 f_1 c_0 e^{-k_{ap} \cdot t}
\]

which after integration yields the fraction of crosslinked tRNA at the plateau level:

\[
F_{450} = \frac{k_1 f_1}{k_3 + (k_1-k_3) f_1}
\]

Taking into account the experimental values of \( k_1 \) and \( k_3 \) yields \( f_1 \) (Fig. 2b)

\[
f_1 = F_{450} / 2.5 - 1.5 F_{450}
\]

Notice that the variations of \( k_{ap} \) are parallel to those of \( f_1 \) as observed in Fig 2c at \( Na^+ \) concentrations lower than 0.2 M.

*To whom correspondence should be addressed

Present addresses: ¹Université Nancy I, Faculté des Sciences Laboratoire d’Enzymologie et de Génie Génétique, BP 239 54506, Vandoeuvre les Nancy Cedex, ²Institut Jacques Monod, Laboratoire de Biochimie de l’Évolution and ³Laboratoire de Biophysique du Museum d’Histoire Naturelle (Paris Ve), INSERM U 201.

REFERENCES
