

# EPR spectroscopic analysis of TAR RNA–metal ion interactions

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## Abstract

Metal ion-induced changes in HIV-1 TAR RNA internal dynamics were determined by the changes in EPR spectral width for TAR RNAs containing spin-labeled nucleotides (U23, U25, U38, and U40). This gave a dynamic signature for each of 10 metal ions studied, which fell into one of three distinct groups. While  $\text{Li}^+$  and  $\text{K}^+$  had little effect on TAR RNA internal dynamics,  $\text{Na}^+$  unexpectedly had a dynamic signature that was similar to  $\text{Ca}^{2+}$  and  $\text{Sr}^{2+}$ , with a decrease in mobility at U23 and U38, little or no change at U25, and an increase in mobility at U40.  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Ba}^{2+}$  had similar effects on U23, U38, and U40, but the mobility of U25 was markedly increased. Our results show that RNA dynamics change upon metal binding to the TAR RNA bulge, indicating that RNA structure adapts to accommodate metal ions of different size and coordination properties.

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RNA performs many diverse roles in biological systems, such as protein assembly, the transfer of genetic information, splicing, and catalysis of chemical reactions [1]. Metal ions are important cofactors in RNA structure and function, facilitating RNA folding, tertiary structure stabilization, and catalysis [2–4]. Several biochemical and biophysical techniques have been used to probe the location and effect of specific RNA–metal ion interactions, such as analysis of the effect of phosphorothioate and phosphonate mutations on catalytic rates [5–8], X-ray crystallography [9–11], and NMR spectroscopy [12,13]. Indirect methods for studying RNA–metal ion interactions include transient electric birefringence [14], laser-induced lanthanide luminescence [15], fluorescence resonance energy transfer [16], and phosphorous relaxation enhancement NMR [17].

Electron paramagnetic resonance (EPR) spectroscopy has been used to study RNA binding to paramagnetic metal ions [18–22]. The interaction of RNA

with metal ions and other molecules has also been studied using EPR spectroscopy of site-specifically spin-labeled RNA [23–25], such as the trans-activation responsive (TAR) RNA, whose complex with the Tat protein is necessary for transcription of full length HIV transcripts [26]. For example, the change in the TAR RNA internal dynamics upon binding Tat-derived peptides [25] and small molecules that inhibit TAR–Tat complex formation [27] was determined. Plotting the change in spectral width, which is associated with changes in spin probe mobility [28], at a number of spin-labeled sites (U23, U25, U38, and U40; Fig. 1A) gave a dynamic signature for each molecule complexed with the RNA. Molecules which bound similarly gave rise to similar dynamic signatures, showing a correlation between RNA dynamics and RNA structure [25,27].

Given the current interest in the interaction of metal ions with RNA [2,4], we have used EPR to investigate the effect of 10 metal ions ( $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Sr}^{2+}$ , and  $\text{Ba}^{2+}$ ) on the TAR RNA internal dynamics. Based on their dynamic signatures, the metal ions were shown to fall into three distinct groups. One of the unexpected findings is that  $\text{Na}^+$  behaves very differently from the monovalent ions  $\text{Li}^+$  and

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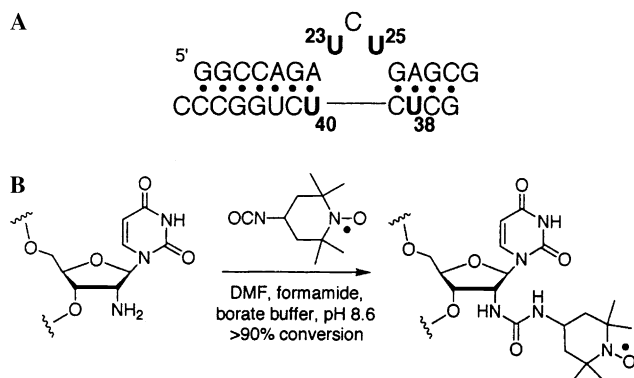


Fig. 1. (A) The TAR RNA, with spin-labeled sites shown in bold. (B) Spin-labeling of RNA at the 2' position using 4-isocyanato TEMPO.

$K^+$ . In fact,  $Na^+$  has a similar effect on the TAR RNA dynamics as the divalent metal ions  $Ca^{2+}$  and  $Sr^{2+}$ . The data presented here show that EPR is a valuable and efficient technique for studying the interactions of metal ions with RNA.

## Materials and methods

**Materials.** Spin-labeled RNAs (Fig. 1A) were prepared as previously described using 2'-amino containing RNAs (Dharmacon Research) labeled with 4-isocyanato TEMPO (Fig. 1B) [25,29]. LiCl, KCl,  $SrCl_2$ , and  $BaCl_2$  were obtained from Aldrich,  $NiCl_2 \cdot 6H_2O$  and  $CdCl_2 \cdot 2\frac{1}{2}H_2O$  from Alfa,  $MgCl_2$  and  $ZnCl_2$  from Baker, NaCl,  $CaCl_2$ , and  $MnCl_2 \cdot 4H_2O$  from Fisher, and  $CoCl_2 \cdot 6H_2O$  from Malinkrodt.

**UV thermal denaturation.**  $A_{260}$  was monitored from 20 to 90 °C at 0.5 °C/min. Three buffers were used: (a) 50 mM NaCl, 5 mM sodium phosphate, 50  $\mu$ M  $Na_2EDTA$ , pH 7.0 (58 mM sodium total); (b) 10 mM potassium phosphate, pH 6.5 (15 mM potassium total); and (c) 1.0 mM potassium phosphate, pH 6.5 (1.5 mM potassium total). RNAs were annealed using a fast cooling process: 90 °C for 2 min, 37 °C for 2 min, and room temperature for 15 min. Melting temperatures were determined as described [29,30].

**EPR spectroscopy.** EPR samples (2.0 nmol spin-labeled RNA in 10  $\mu$ l 20% aqueous sucrose/1.0 mM potassium phosphate buffer at pH 6.5, including metal chloride salts of appropriate concentration) were annealed as previously described [25]. To compare the effects of monovalent and divalent metal ions, all samples were prepared with the same ionic strength, determined by the equation  $I = 0.5 \sum c_i z_i^2$ , where  $I$  is the ionic strength,  $c$  is ion concentration, and  $z$  is the charge of the ion, yielding concentrations of 10 mM divalent metal ions and 30 mM monovalent metal ions. X-band EPR spectra were digitally recorded on a Bruker EMX 300 spectrometer with TE102 cavity using the following experimental parameters: 8 mW power, 3355 G center field, 20.48 ms time constant, 40.96 ms conversion, 42 s sweep time, 1024 points, 100 kHz modulation frequency, 1.0 G modulation amplitude, 110 G sweep width, typically 100 scans, and  $273.2 \pm 0.2$  K. EPR spectra in the presence of  $Mn^{2+}$  were acquired as above and the nitroxide spectrum was obtained by spectral subtraction of the unbound paramagnetic  $Mn^{2+}$  component. Changes in EPR spectral width ( $2A_{zz}$ ), which spans the crest of the low field peak to the trough of the high field peak, were measured in Gauss and plotted as a function of spin-labeled position to give dynamic signatures. The error in measuring the spectral width is  $\pm 0.3$  G.

## Results

### Selection of ionic conditions

Background metal ions can potentially mask important contributions of the ion being studied and therefore, aqueous conditions void of metal ions are optimal for the study of RNA–metal ion interactions. However, RNA requires metal ions for secondary structure formation and thus, conditions with a minimal amount of monovalent metal ions that promote stable RNA duplex formation were selected. As in other RNA–metal ion studies [23], potassium ions were chosen over sodium ions due to their large ionic size, which makes them less likely to form specific interactions. TAR RNA UV thermal denaturation data collected at a concentration of 1.5 mM  $K^+$  showed a cooperative melting transition and revealed a melting temperature of 47.3 °C (data not shown), only 3.4 °C lower than that previously measured at 58 mM  $Na^+$  [29], demonstrating that the RNA forms a stable duplex. Furthermore, all four TAR RNA samples produced EPR spectra indicative of duplex formation [25] under the new buffer conditions. For example, the U40 TAR RNA duplex had a wider spectral width ( $2A_{zz}$ ) than the U40 single strand (Fig. 2), demonstrating reduced mobility of U40 upon base-pairing, consistent with previous data on structure-dependent mobility of nucleotides [29].

### Monitoring the effect of $Mg^{2+}$ concentration on the TAR RNA by EPR

To investigate the concentration dependence of metal ions on the internal dynamics of the TAR RNA, chan-

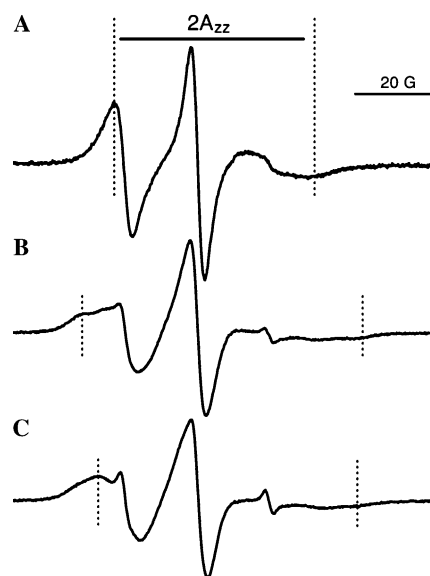


Fig. 2. EPR spectra of spin-labeled U40 single strand (A) and U40 TAR RNA duplex in the absence (B) and presence (C) of 10 mM  $Mg^{2+}$ . Dotted lines indicate the width ( $2A_{zz}$ ) of each spectrum.

ges in the EPR spectra of spin-labeled U40 TAR RNA, which showed the largest spectral change in preliminary experiments, were monitored as a function of increasing  $\text{Mg}^{2+}$  concentration.  $\text{Mg}^{2+}$  was chosen because it is one of the most prolific RNA–metal ion cofactors [31] and has previously been shown to bind to the TAR RNA [14,25,32]. The EPR spectral width decreased up to 2 mM  $\text{Mg}^{2+}$  (Fig. 3). These data are consistent with site-specific  $\text{Mg}^{2+}$ -binding at 2 mM  $\text{Mg}^{2+}$ , indicating a  $K_d \sim 1$  mM, similar to that measured by  $^{19}\text{F}$  NMR [32]. Further, nonspecific RNA–metal ion interactions do not change the spectral width, but contribute to minor changes in line shape up to 10 mM (data not shown). To account for changes in both spectral width and line shape, 10 mM  $\text{Mg}^{2+}$  was chosen as the reference point, especially since this concentration is within the range of biological concentration of  $\text{Mg}^{2+}$  (for example, 2 mM  $\text{Mg}^{2+}$  in blood plasma and 26 mM  $\text{Mg}^{2+}$  in intercellular fluid). Titration of U38 TAR RNA with  $\text{Mg}^{2+}$  showed similar results (data not shown).

#### EPR spectroscopy of TAR RNA in the presence of different metal ions

A summary of changes in TAR RNA internal dynamics, as indicated by changes in spectral width, at

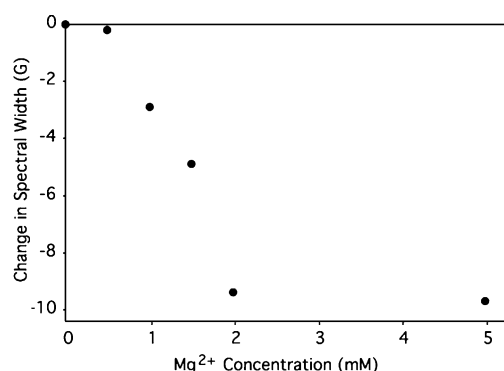


Fig. 3. Titration of U40 spin-labeled TAR RNA with magnesium.

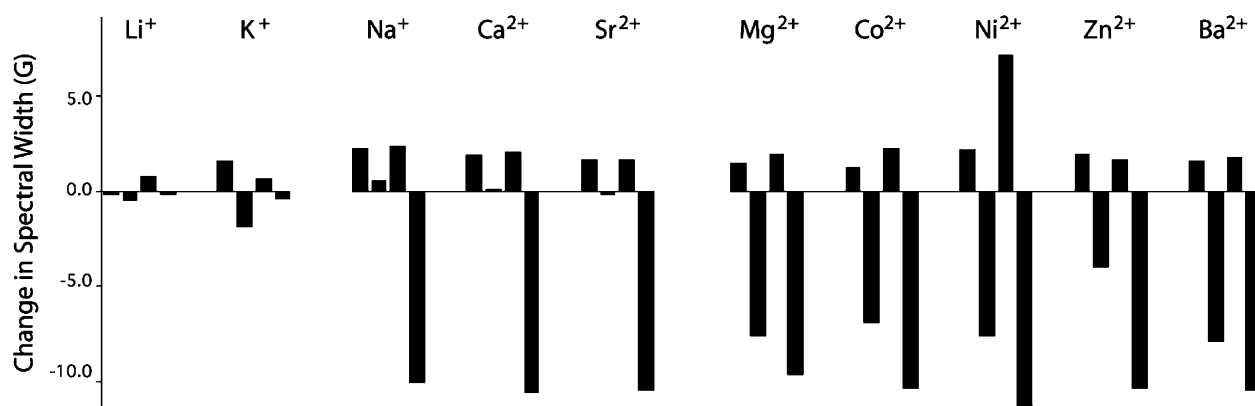


Fig. 4. Dynamic signatures of TAR RNA complexed with metal ions. Change in spectral width was plotted as a function of spin-label position (left to right for each metal ion, U23, U25, U38, and U40) to give the dynamic signature for each metal ion. All spectra were recorded at the same ionic strength.

U23, U25, U38, and U40 is shown for each metal ion in Fig. 4.  $\text{Li}^+$  and  $\text{K}^+$  had a minor effect on the internal dynamics of the TAR RNA.  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Sr}^{2+}$  gave similar dynamic signatures, with an increase in spectral width at U23 and U38, little or no change at U25, and a decrease at U40. In addition, all three metal ions induced similar changes in line shape at individual positions (data not shown).  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Ba}^{2+}$  yielded a different set of dynamic signatures with an increase in spectral width at U23 and U38 and a decrease at U25 and U40. Interestingly, the TAR RNA precipitated upon incubation with  $\text{Cd}^{2+}$ , thereby immobilizing the nitroxide reporter group to produce near powder pattern spectra, which eliminated meaningful comparison with other metal ion dynamic signatures (data not shown). The nitroxide component of the EPR spectra of spin-labeled TAR RNAs in the presence of the paramagnetic  $\text{Mn}^{2+}$  was solved by spectral subtraction (see Materials and methods) but displayed extremely weak signal at all positions, which precluded the determination of its dynamic signature.

An alternate method for analyzing the EPR data is to determine the rotational correlation times ( $\tau_r$ ) of the nitroxides. The calculation of rotational correlation times were based on a Brownian diffusion model (the “ $\Delta S$  method”), which utilizes the changes in the spectral width ( $\Delta A_{zz}$ ) [28]. The resulting values ranged from 2.3 to 6.4 ns, using the parameters associated with a 3 G center line width (data not shown). Plotting the change in rotational correlation time ( $\Delta\tau_r$ ) as a function of spin-labeled position for each metal ion yielded a plot almost identical to Fig. 4 (data not shown).

#### Discussion

High resolution structural methods such as crystallography and solution NMR often require metal ion concentrations much higher than physiological conditions in order to solve complex RNA structures. To

study specific RNA–metal ion interactions, techniques more sensitive to subtle changes in RNA structure and dynamics under biological metal ion concentrations are required.  $^{19}\text{F}$  NMR [32,33] and EPR spectroscopy [23,24] have shown promise in the detection of metal ion-induced changes in RNA structure and dynamics. For example, it has been shown by EPR spectroscopy that under crystal structure-like ionic conditions,  $\text{Ca}^{2+}$  has a similar effect on the TAR RNA internal dynamics as  $\text{Na}^+$  [25]. This indicates that the structural differences between the crystal structure [34] and solution NMR structure [35] of the TAR RNA were not specifically due to the  $\text{Ca}^{2+}$  ions found in the TAR crystal structure. The focus of the work presented here is to determine the effects of a variety of metal ions, that have been used in the study of RNA structure and function, on TAR RNA dynamics.

The EPR data are summarized in Fig. 4, where changes in spectral width at individual sites are plotted as a function of position to yield a dynamic signature for each set of conditions. Increases in spectral width ( $2A_{zz}$ ) have been shown to correlate with decreases in mobility of the nitroxide spin probe [28]. Only minor changes were observed for  $\text{Li}^+$  and  $\text{K}^+$ . Interestingly,  $\text{Na}^+$  behaved differently than the other monovalent metal ions, producing a dynamic signature in line with divalent metal ions of similar ionic radii ( $\text{Na}^+$ , 0.97 Å;  $\text{Ca}^{2+}$ , 0.99 Å; and  $\text{Sr}^{2+}$ , 1.12 Å) and  $\text{pK}_a$ s of the associated metal–aqua complexes ( $\text{Na}^+$ , 14.8;  $\text{Ca}^{2+}$ , 12.6; and  $\text{Sr}^{2+}$ , 13.2). With the exception of  $\text{Ba}^{2+}$  (1.34 Å and  $\text{pK}_a$  13.4), the metal ions in the third group all have similar ionic radii ( $\text{Mg}^{2+}$ , 0.66 Å;  $\text{Co}^{2+}$ , 0.72 Å;  $\text{Ni}^{2+}$ , 0.69 Å; and  $\text{Zn}^{2+}$ , 0.74 Å) and  $\text{pK}_a$ s of their metal–aqua complexes ( $\text{Mg}^{2+}$ , 11.4;  $\text{Co}^{2+}$ , 9.6;  $\text{Ni}^{2+}$ , 10.6; and  $\text{Zn}^{2+}$ , 9.0), which differ from those in the first or second group.  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  showed different effects on the RNA internal dynamics and structure, which is similar to previous EPR data under crystal structure-like metal ion concentrations [25]. These results are also consistent with  $^{19}\text{F}$  NMR data which showed that  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  induced different  $^{19}\text{F}$  chemical shifts of 5-fluoro modified uridine nucleotides at several of the positions studied here [32]. It is worth noting that although the  $\text{Ni}^{2+}$  dynamic signature has the same pattern as the metals in group three, there are some differences, primarily in the magnitude of change at U38.  $\text{Cd}^{2+}$  caused the RNA to precipitate and the paramagnetic  $\text{Mn}^{2+}$  caused the nitroxide EPR signals to almost completely disappear. The most plausible explanation for the absence of a strong, definitive EPR signal after subtracting the free  $\text{Mn}^{2+}$  spectrum is that  $\text{Mn}^{2+}$  binds close to the nitroxides (within 20 Å), broadening the spectra through electron–electron dipolar coupling. As a result, we were unable to obtain a dynamic signature for  $\text{Mn}^{2+}$ .

The dynamic signatures for the second and third groups are similar, but differ in the dynamics of U25,

which is located in the flexible bulge. This is in contrast to previous observations in studies of small molecule binding to the TAR RNA, where compounds that bound differently to the RNA had strikingly different signatures [27], and indicates that the metal ions in groups two and three do not have dramatically different effects on the overall RNA structure and dynamics. The largest change in dynamics for groups two and three is the increase in mobility of U40, yielding a rotational correlation time similar to that of U38 (data not shown). Structural studies indicate that increased mobility of U40 could be associated with helical stacking: the crystal structure of the TAR RNA, solved in the presence of  $\text{Ca}^{2+}$  (group two), exhibits collinear helical stacking [34], whereas a solution NMR structure, solved in the presence of  $\text{K}^+$  (group one), shows a, ca. 50°, bend between the two helical regions [35]. However, EPR data show that the mobility of U40 decreases upon binding to derivatives of the Tat protein [25], which results in coaxial stacking of the two helices [36]. Therefore, the change in the mobility of U40 could indicate changes in the global conformation of the RNA or changes in flexibility at the interface of the two helices, where U40 is located.

Although we do not fully understand the effects of the metal ions on the RNA structure, our results clearly show that the TAR RNA dynamics and structure change upon metal ion binding. Furthermore, EPR spectroscopy effectively identifies similarities and differences between individual metal ions that bind to the TAR RNA. These data show that EPR is a sensitive technique for studying RNA–metal ion interactions and that EPR data can provide information about important principles of molecular recognition.

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