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ARTICLE

Rapid NMR screening of RNA secondary structure and binding

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Abstract Determination of RNA secondary structures by NMR spectroscopy is a useful tool e.g. to elucidate RNA folding space or functional aspects of regulatory RNA elements. However, current approaches of RNA synthesis and preparation are usually time-consuming and do not provide analysis with single nucleotide precision when applied for a large number of different RNA sequences. Here, we significantly improve the yield and 3′ end homogeneity of RNA preparation by in vitro transcription. Further, by establishing a native purification procedure with increased throughput, we provide a shortcut to study several RNA constructs simultaneously. We show that this approach yields µmol quantities of RNA with purities comparable to PAGE purification, while avoiding denaturation of the RNA.

Keywords NMR spectroscopy · RNA secondary structure · In vitro transcription · Riboswitch RNA · High throughput method

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Introduction

Detailed secondary structure determination of RNA by NMR spectroscopy is typically a time-consuming procedure and includes DNA template preparation, RNA synthesis, RNA purification and subsequent NMR-based structure analysis. Due to the comparably large amount and high purity of RNA required for analysis, all of these steps can be challenging during the preparation of an RNA sample for NMR spectroscopy.

Current approaches for in vitro transcription are based on DNA templates derived from either linearized plasmids, or PCR (Milligan and Uhlenbeck 1989; Pokrovskaya and Gurevich 1994; Beckert and Masquida 2011). Both of these DNA templates are suitable to supply sufficient amounts of DNA for large scale transcriptions, yielding milligram quantities of RNA. However, standard techniques require the presence of certain nucleotides at the two or three first transcribed nucleotides on the 5′ terminus adjacent to the promotor sequence (Stage-Zimmermann and Uhlenbeck 1998). Further, the most common T7 RNA polymerase is prone to generate inhomogeneous 3′ ends during run-off transcription (Milligan et al. 1987; Draper et al. 1988; Krupp 1988; Pokrovskaya and Gurevich 1994). Besides leading to deterioration of NMR spectral quality, the inhomogeneous addition of non-native nucleotides at the 3′ terminus cannot be accepted in experiments if exact sequence-dependent folding or binding needs to be investigated. The problem is usually overcome by introduction of self-cleaving ribozyme cassettes 5′ or 3′ of the desired RNA sequence (Been et al. 1992; Chowrira et al. 1994; Price et al. 1995; Ferre-D'Amare and Doudna 1996; Birikh et al. 1997). However, presence of these ribozymes necessitates additional purification steps, decreases the efficiency of incorporation of isotope-labeled nucleotides

for NMR spectroscopy and can lead to significant separation problems between target RNA and ribozyme during purification. Alternatively, improved 3′ end homogeneity can be achieved if the RNA is transcribed from PCR products that are 2′-*O*-Methyl-modified at the last two nucleotides of the 5′ end (Kao et al. 1999). While in vitro transcription is the method of choice for generating RNA samples, many protocols still produce insufficient amounts, in particular for kinetic RNA folding experiments monitored by NMR (Fürtig et al. 2007; Buck et al. 2009).

Following transcription, the most common methods for RNA purification are polyacrylamide gel electrophoresis (PAGE) (Petrov et al. 2013), size exclusion chromatography (SEC) (Lukavsky and Puglisi 2004; Kim et al. 2007), ion-pair reversed phase HPLC (Azarani and Hecker 2001) or weak anion-exchange FPLC (Easton et al. 2010). All of these methods have a limited-to-low throughput and are inefficient for rapid screening procedures. Furthermore, in most cases (PAGE and HPLC) the RNA is denatured during the purification procedure and therefore the conformation adopted during transcription is not maintained. This problem also arises in structural probing techniques, which is one of the few approaches that allows simultaneous structural analysis of different samples. Affinity purification of RNA is a new generation of techniques for native RNA purification using an affinity tag to immobilize the RNA on an affinity column that is later removed by ribozyme-catalyzed cleavage with homogeneous 3′ and 5′ end (Kieft and Batey 2004; Batey and Kieft 2007; Keel et al. 2009; Di Tomasso et al. 2011, 2012; Salvail-Lacoste et al. 2013; Di Tomasso et al. 2014; Batey 2014). However, ribozyme and affinity tag comprise over 200 nt causing a significant decrease in the final yield of the desired RNA (Easton et al. 2010). Furthermore, these methods have to be improved for each sample in terms of cleavage conditions and ratios.

Here, we present a method for simultaneous screening of RNA secondary structure of several RNA constructs by NMR, which can be prepared within 2 days. We develop this new method for the parallel analysis of multiple RNA riboswitch structures. For transcriptional riboswitches, their regulatory function requires binding to transcription intermediates but not to full-length sequences (Wickiser et al. 2005). In these cases, ligand binding affinity of the RNA varies with the length of the construct. It is therefore imperative to screen numerous constructs to identify structures that are competent to bind their cognate ligand.

We benchmark our method to investigate ligand-binding properties of the 2′dG-sensing riboswitch aptamer domain from *Mesoplasma florum* (Kim et al. 2007; Wacker et al. 2011; Pikovskaya et al. 2011) with increasing RNA chain length. We use PCR for simultaneous amplification of the DNA template for transcriptional intermediates of varying length, and demonstrate that run-off transcriptions with T7 RNAP can yield highly homogeneous RNA if 2′-methoxy modified DNA is used in combination with dimethyl sulfoxide (DMSO) as a cosolvent. We furthermore show that the screening process can be accelerated by direct buffer exchange and concentration of the transcription mixture in centrifugal concentrators, while avoiding time-consuming purification steps and denaturation of the RNA. We compare this approach to RNA transcribed directly in the NMR tube (Okui and Kawai 2015) and show that the buffer exchange method produces NMR spectra of superior quality that are comparable to spectra obtained from purified samples. The mRNA screening procedure on the 2′dG aptamer domain clearly delineates the requirement on sequence length for ligand binding: Only in the presence of a 4 bp long P1 helix, ligand binding can take place. Our approach therefore combines several improved methodologies with respect to RNA synthesis and purification to facilitate rapid and detailed analysis of RNA structures using NMR spectroscopy.

Materials and methods

Transcription template

Transcription templates were prepared by polymerase chain reaction from a plasmid containing the full length sequence of the 2′dG-sensing riboswitch. Transcription templates for dGsw^{75}–dGsw^{80} and dGsw^{85} were amplified by varying the reverse primers. PCR was performed according to standard protocols from NEB (0.2 μM of each Primer, 0.2 ng/μL DNA template, 200 μM dNTPs) using Phusion® High-Fidelity DNA Polymerase. For the DNA template preparation for rapid screening of dGsw^{75}–dGsw^{80} the following primers were used (2′-*O*-Me modified nucleotides are placed within square brackets []):

Construct	Forward primer	Reverse primer
dGsw^{75}	TAA TAC GAC TCA CTA TAG G	[AG]T CTC CAA ATA GGT TTG AAG
dGsw^{76}		[UA]G TCT CCA AAT AGG TTT GAA G ATA GTC TCC AAA TAG GTT TGA A ATA GTC TCC AAA TAG GTT TGA A
dGsw^{77}		[AU]A GTC TCC AAA TAG GTT TGA A
dGsw^{78}		[UA]T AGT CTC CAA ATA GGT TTG A
dGsw^{79}		[UU]A TAG TCT CCA AAT AGG TTT G
dGsw^{80}		[CU]T ATA GTC TCC AAA TAG GTT T

RNA preparation

RNA fragments were prepared by in vitro transcription with T7 RNA polymerase from PCR products. The PCR mixture was directly applied for transcription without further purification. Transcriptions were incubated for 16 h at 37 °C in transcription buffer (200 mM Tris·HCl, pH 8.1) with 2 mM spermidine, 5 mM of each NTP, 10 mM $Mg(OAc)_2$, 20 mM dithiothreitol (DTT), 20 % (v/v) of DMSO, 0.2 u/μL of inorganic pyrophosphatase (NEB), 2 % (v/v) of the PCR mixture and 144 nM T7 RNA polymerase (homemade) (Guillerez et al. 2005). Unlabeled NTPs were purchased from Carl Roth GmbH (Karlsruhe), ^{15}N labeled NTPs and ^{15}N,^{13}C labeled 2′-deoxyguanosine from Silantes (Munich).

NMR samples were prepared simultaneously in a three-step approach including PCR for generating the DNA templates, transcription and RNA purification. Transcription templates for $dGsw^{75}$–$dGsw^{80}$ of RNAs were generated with PCR by shifting the reverse primers by one nucleotide. Transcriptions for NMR screening ($dGsw^{75}$–$dGsw^{80}$) were performed in a 5 mL scale according to the protocol described above. For buffer exchange and washing steps, centrifugal concentrators with a molecular weight cuf-off of 3.000–5.000 (Vivaspin® 20 from Sartorius AG, Goettingen) were used. The transcription mixture was first washed with 5 mL of transcription buffer to remove phosphate produced by inorganic pyrophosphatase during the transcription. Subsequently, the mixture was concentrated to 1 mL and washed with 60 mL ($dGsw^{75}$–$dGsw^{79}$) or 120 mL ($dGsw^{80}$) of NMR buffer (25 mM potassium phosphate, pH 6.2) in 5 mL steps, which corresponds to a dilution factor of $<10^{-8}$ and $<10^{-16}$, respectively. Following buffer exchange the samples were concentrated to Shigemi tube sample volume (300 μL).

Transcriptions in NMR tubes were performed in a 500 μL scale in 20 % DMSO-d_6. For direct assessment of the transcription in the NMR tube, the pH was adjusted to 5.8 by addition of hydrochloric acid without adjusting the buffer composition. Stability tests were performed by incubation at 37 °C for 6 days.

Purity and homogeneity of RNA transcripts were analysed by polyacrylamide gel electrophoresis (8–20 % 29:1 (w/w) acrylamide/bisacrylamide, 7 M urea). Gels were stained with GelRed™ and visualized on a Gel iX imager (Intas).

PAGE purification was performed with 10 % polyacrylamide gels (29:1 (w/w) acrylamide/bisacrylamide, 7 M urea). The RNA was visualized by UV shadowing (254 nm), excised from the gel and eluted with 0.6 M NaOAc (pH 5.5). Eluted RNA was first precipitated with five volumes of ethanol (−80 °C) and then twice with five volumes of 2 % (w/v) $LiClO_4$ in acetone. Purified RNA was refolded by thermal denaturation of the RNA at high concentrations (0.2–0.5 mM) followed by a tenfold dilution with water (0 °C) and incubation on ice for 1 h. Folded RNA was exchanged into NMR buffer.

NMR spectroscopy

NMR experiments were recorded on Bruker AV800 and AV900 NMR spectrometers equipped with a 5-mm z-axis gradient TXI-HCN cryogenic probe and on a Bruker AV600 spectrometer equipped with 5-mm z-axis gradient TCI-HCN cryogenic probe. Data were processed using the software Topspin 2.1 and 3.2 (Bruker Biospin). [^1H–^{15}N]-TROSY and –HSQC spectra were analysed with the software Sparky 3.114 (Goddard and Kneller). PAGE-purified NMR samples and buffer exchanged samples were prepared in NMR buffer with 5 % D_2O added, while samples transcribed in the NMR tube were prepared in transcription buffer in 20 % DMSO-d_6. 1D ^1H-NMR spectra were recorded with a jump-return echo experiment (Sklenář and Bax 1987). [^1H–^{15}N]-HSQC spectra were recorded implementing the soft WaterGATE water suppression scheme (Piotto et al. 1992). [^1H–^{15}N]-TROSY experiments were recorded with modifications proposed by Lescop et al. (2010) and Favier and Brutscher (2011).

Results and discussion

Fast synthesis of transcriptional intermediates in NMR scale

The overall procedure for NMR sample preparation is shown in Fig. 1a. Since all three steps can be performed for several RNAs simultaneously, this procedure facilitates RNA preparation for secondary structure analysis of a multitude of RNAs within 2 days or ∼24 h that can be parallelized for a large number of sequences followed by NMR acquisition of 1D and 2D NMR data. The rate-limiting step in this procedure is an infrastructural one, namely the availability of centrifuges required for buffer exchange. Our overall aim is to rapidly analyse the secondary structure of a multitude of transcriptional intermediates by NMR spectroscopy with single nucleotide resolution. Therefore, we applied the described procedure to investigate the increase in ligand binding affinity of the 2′dG-sensing riboswitch from *Mesoplasma florum* with increasing RNA length by NMR. In particular, ligand binding was monitored in the absence of P1 ($dGsw^{75}$) up to a stabilized P1 helix ($dGsw^{80}$) in steps of one nucleotide (Fig. 1b).

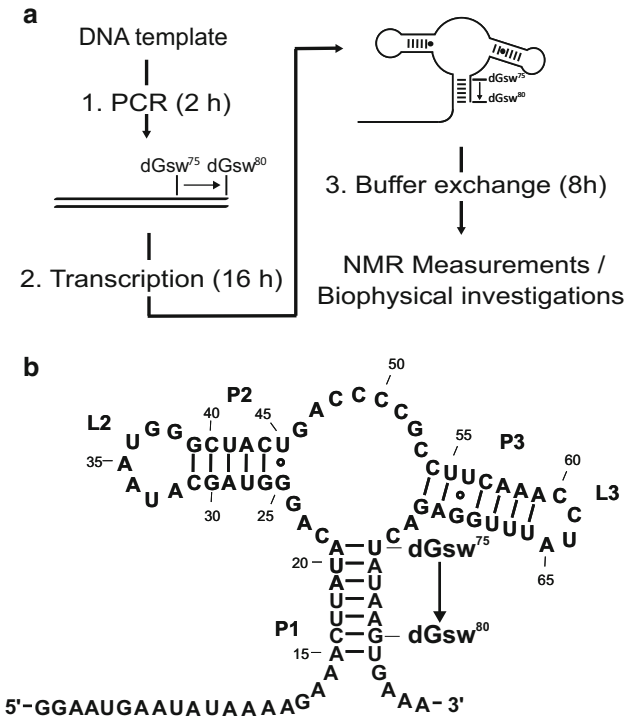

Fig. 1 **a** Schematic representation of the three-step screening approach. DNA templates are prepared by PCR and subsequent transcriptions are performed overnight. The transcription mixture is then buffer exchanged and concentrated in a centrifugal device, and directly submitted to NMR measurements. **b** Secondary structure of the native 2′dG-sensing riboswitch aptamer domain (dGsw^{85}) modified at the 5′-end with GG to increase the transcription efficiency. Transcriptional intermediates rapidly screened for ligand binding by NMR are highlighted (dGsw^{75}–dGsw^{80})

Improvement of transcription yield and homogeneity

The aim of this work is to develop a method that allows rapid screening of tens of different mRNAs with varying length by biophysical methods (NMR, CD, ITC, UV melting). For this approach to work, high transcription yields, homogeneity at single nucleotide level and purity for each RNA are imperative. When comparing various conditions employing the abovementioned approaches (cosolvent DMSO, 2′-methoxy modified primers) for increased yield and improved 3′ end homogeneity, we found that DMSO did not only increase transcription yield as reported previously (12 vs 3 % incorporation efficiency) (Strätling 1976; Juang and Liu 1987; Chen and Zhang 2005), but also had an impact on 3′ end homogeneity (Fig. 2). For the template used in this study, 3′ end homogeneity proved to be difficult under standard conditions. In addition to the expected +1 and +2 nucleotide transcripts, we also found longer transcripts (>4 nt) including very large fragments that remain in the wells. The amount of these longer byproducts exceeded that of the desired transcript. However, when titrating DMSO in the transcription mixture to improve the yield, we also found that in these mixtures the product exhibited a high degree of homogeneity (>75–95 %). The effect even exceeded that of 2′-*O*-Me-primers (Kao et al. 1999) with respect to the greater than +4 nt transcripts, while 2′-methoxy modified primers seem to have a larger effect on +1 and +2 nucleotide transcripts.

In order to investigate whether the increase in 3′ end homogeneity by DMSO is sequence-correlated, we compared different RNA transcripts. In particular, we investigated sequences that contain large stretches of Poly-U and/or Poly-A, which in our experiments resulted in products with the highest degree of heterogeneity from standard run-off transcriptions (Supplementary Figure S1 and S2). We assume that the transcription of RNAs longer than the template sequence is due to Poly-A and -U stretches in the DNA/RNA duplex, since incorporation of a G and C dramatically reduces non-DNA templated nucleotide addition. This large amount of non-DNA templated nucleotide addition is completely prevented in the presence of DMSO, while only some remaining unspecific nucleotide addition (n + 1) can be detected.

We therefore report—to the best of our knowledge—for the first time that DMSO reduces transcription product inhomogeneity for T7-based transcriptions. Previous work on the influence of DMSO on in vitro transcriptions focused on the increase in yield and the cofactor induced transcription termination in the presence of DMSO (Strätling 1976; Juang and Liu 1987; Chen and Zhang 2005). Optimizations for the 2′dG riboswitch aptamer show that for this particular sequence, 20 % of DMSO is required for optimal 3′ end homogeneity. Only for constructs dGsw^{75}, dGsw^{76}, and dGsw^{80} we detected a minor n + 1 fraction in the presence of DMSO (Fig. 2c). We therefore chose to combine 20 % DMSO with 2′-methoxy modified transcription templates. Figure 2c shows that the combination of both DMSO- and 2′-methoxy-modified DNA eliminates n + 1 activity also for dGsw^{75}, dGsw^{76} and dGsw^{80} and produces highly homogeneous RNA from run-off transcriptions.

As this approach was applicable for several RNA constructs studied, it greatly facilitated our approach of using a multiplex-PCR approach to generate DNA transcription templates of one nucleotide difference in length. These templates were used directly from the PCR mixture, and the high precision of 3′ end formation during transcription translates into highly pure RNA samples. This precision allows us to transcribe various transcriptional intermediates at single nucleotide resolution simultaneously and directly from dsDNA PCR products without altering the initial template, which would be required if 3′-ribozymes were used.

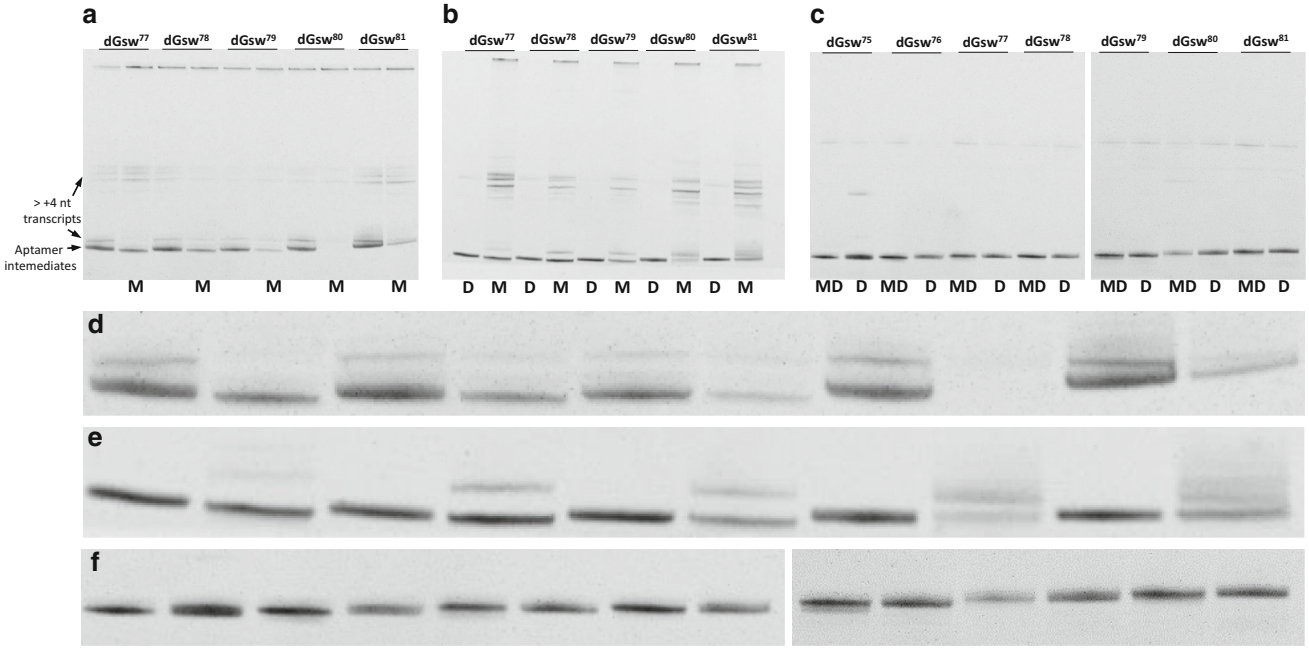

Fig. 2 20 % polyacrylamide gels investigating the 3′ end homogeneity of RNA transcripts. Comparison of run-off transcriptions from: **a** 2′-methoxy modified DNA (M) and standard PCR products, **b** 2′-methoxy modified DNA (M) and standard PCR products in 20 % of DMSO (D), **c** standard PCR products in 20 % of DMSO and from 2′-methoxy modified DNA in 20 % of DMSO (MD), **d**, **e**, **f** enlarged section of the target sequences shown in **a**, **b** and **c**

RNA purification

The aforementioned strategy improves the homogeneity of transcribed RNA and effectively prevents the formation of side products during transcription and therefore allows us to avoid time-consuming purification procedures. Instead, we directly exchanged the transcription mixture into NMR buffer using centrifugal devices as a shortcut to accelerate the overall process. Provided that the sample is sufficiently washed, this process can completely remove NTPs and transcriptional additives including Mg^{2+} and spermidine. These additives are known to interact with the RNA, increase the thermal stability of the RNA (Cole et al. 1972; Tabor and Tabor 1984) and also alter the NMR imino proton pattern. These alterations in the imino proton pattern have been observed previously where transcriptions were directly performed within the NMR tube and measured (Okui and Kawai 2015). Components that cannot be removed during the procedure are enzymes (T7 RNA polymerase, inorganic pyrophosphatase) and the DNA template. However, the concentration of these components is below the NMR detection limit (T7 RNAP: 2.4 μM, DNA: <8.5 μM), and their suppression can be further improved when using isotope-labeled nucleotide triphosphates. Furthermore, imino protons are sufficiently isolated to separate RNA signals from signals of enzymes and other organic molecules.

Our results show that the shorter constructs (dGsw^{75}–dGsw^{79}) yield comparable NMR spectra to purified dGsw aptamers by washing with 60 mL of NMR buffer. For constructs with increasing P1 stability (dGsw^{80}) we could, however, detect changes in the imino proton pattern. These changes mostly involve line broadening and additional signals, which could be assigned to tertiary interactions that appear as a result of preorganization within the aptamer domain towards the ligand bound state (Buck et al. 2010; Wacker et al. 2011). We assume that these signals are correlated to RNA interactions with residual Mg^{2+} or spermidine, which bind more strongly to construct dGsw^{80} due to the increase in P1 stability. We could, however, show that these signals do not appear after increase of the washing volume to 120 mL (Supplementary Figure S3).

Comparison of NMR spectra of purified RNA with buffer exchanged transcriptions and RNA transcribed in the NMR tube without purification

Prior to conducting rapid mRNA secondary structure screening as described previously, we compared NMR spectra of the 2′dG riboswitch aptamer domain of a PAGE-purified sample with a buffer exchanged and concentrated sample (dGsw^{85}). For practical reasons, the PAGE-purified RNA does not contain the first two 5′-Gs as shown in Fig. 1b since it was previously prepared as a fusion product

with a 5′-hammerhead ribozyme. Apart from minor line broadening, the imino imprint in 1D NMR spectra of purified and buffer-exchanged RNA can be considered identical (Fig. 3a). The remaining enzymes in the transcription mixture, which remain present throughout washing, are not affecting the analysis as depicted in Fig. 3. However, more detailed NMR studies might require removal of those. Phenol extracting the proteins prior to washing might be an option, however, the fold of the RNA might be affected by this step. When comparing the S/N ratio of PAGE purified with buffer exchanged dGsw^{85}, buffer exchange of a 5 mL transcription roughly yields a 600 µM NMR sample (i.e. 0.2 µmol).

As an alternative method we also investigated NMR spectra obtained from direct transcription in the NMR tube. It was reported recently that the generation of RNA during transcription can be monitored directly within the NMR tube, even though the authors did not investigate the

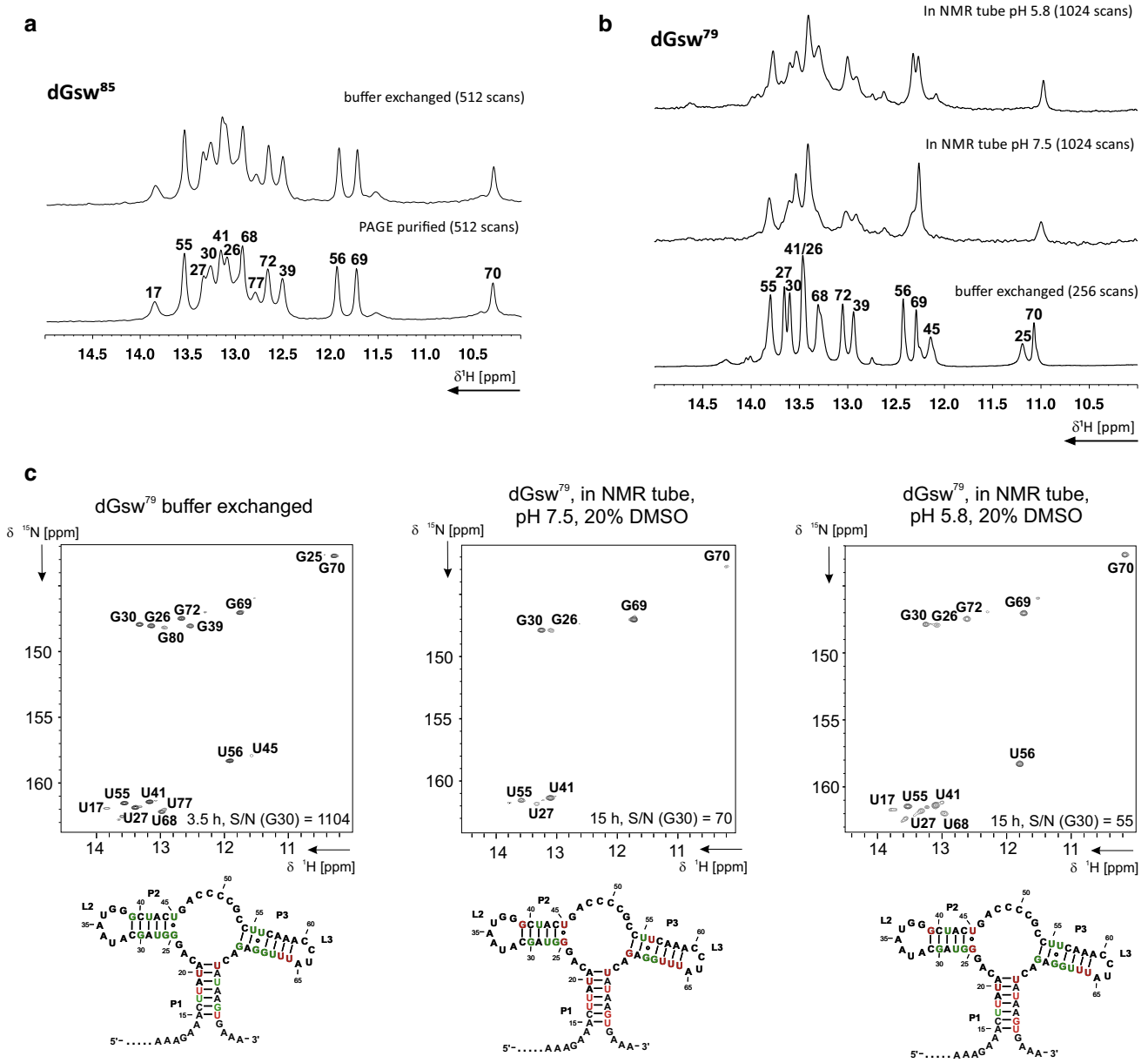

Fig. 3 **a** Comparison of ^{1}H NMR spectra of dGsw^{85} buffer exchanged and PAGE purified recorded at 600 MHz. **b** Comparison of ^{1}H NMR spectra obtained from buffer exchanged dGsw^{79} with in NMR tube transcribed dGsw^{79} at pH 7.5 and 5.8 recorded at 800 MHz. **c** ^{1}H–^{15}N-TROSY spectra obtained from buffer exchanged dGsw^{79} (3.5 h), in NMR tube transcribed dGsw^{79} at pH 7.5 and 5.8 (15 h). Residues of detectable imino protons are highlighted in *green* and residues of absent imino protons in helical segments are highlighted in *red*. The spectra were recorded at 800 MHz. All measurements were performed at 298 K

structural integrity of the RNA (Okui and Kawai 2015). Comparison of ^{1}H and ^{15}N-TROSY spectra of buffer exchanged dGsw^{79} and in-NMR-tube transcribed dGsw^{79} show that in addition to significant line broadening, imino proton signals for U77, U17 (P1), G39, G25, U45 (P2), G72, U68, and U56 (P3) are missing in the NMR tube transcription sample (Fig. 3b, c). We hypothesize that the pH value of 7.5 of the transcription mixture is responsible for the absence of these signals since an increase in pH is generally accompanied with an increase in imino proton solvent exchange. In order to verify this assumption we repeated the experiment and adjusted the pH to 5.8. At pH 5.8, signals assigned to helix P3 (G72, U68, U56) and U17 (P1) reappear, while signals from the closing base-pairs including G25, U45, G39 still cannot be detected. In addition, the low signal-to-noise ratio of spectra obtained from in NMR tube transcriptions requires significantly more scans in comparison to the buffer exchanged sample. The buffer exchanged sample was recorded with an eight times smaller number of scans (64:512) and yields a S/N value 15 times larger in comparison with the in NMR tube transcription. Furthermore, chemical shift perturbations can be detected when comparing purified and in-NMR-tube transcribed dGsw^{79}. These perturbations could be caused either by the different buffer composition or the presence of 20 % of DMSO. While the sample preparation of in NMR tube transcription may be faster than buffer exchange, the low S/N leads to a significant increase in required NMR measurement time. In addition, the buffer exchange procedure yields NMR spectra that are comparable to purified samples and homogeneity with regard to low molecular weight additives (Mg^{2+}, spermidine) can be achieved. We therefore conclude that for large-scale screening of RNA secondary structures, buffer exchange of the transcription mixture is mandatory and represents the superior method compared to transcriptions performed directly in the NMR tube.

Ligand binding by transcriptional intermediates of the 2′dG-sensing riboswitch aptamer domain

According to transcription termination assays, the 2′dG-sensing riboswitch is assumed to be regulating on the level of transcription (Kim et al. 2007). It has been shown previously that particularly in case of transcriptional riboswitches, ligand-binding by the aptamer domain is a co-transcriptional event (Wickiser et al. 2005). We therefore show the viability of the rapid NMR screening method by monitoring at which time during transcription the aptamer domain is sufficiently stabilized to bind its cognate ligand at single nucleotide resolution. To this end, we screened the secondary structure of six transcriptional intermediates as described previously (Fig. 1b) in both presence and absence of 2′dG. The RNA samples were first titrated with 3 mM Mg^{2+} to promote ligand binding and then with 1 mM 2′dG. The RNA was prepared in natural isotope abundance, while the ligand was applied in ^{15}N-isotope labeled form. The imino pattern of the RNA was monitored upon addition of each component. After addition of ligand, ^{15}N-HSQC spectra were recorded to resolve the ligand imino proton signal and to investigate the degree of ligand binding.

1D ^{1}H-NMR spectra obtained from rapid screening of dGsw^{75}–dGsw^{80} all exhibit similar imino proton patterns (Fig. 4a). All spectra mainly show signals corresponding to helices P2 and P3. This is expected, since helix P1 is not stabilized enough in absence of Mg^{2+} and ligand to decelerate the imino proton solvent exchange sufficiently for NMR detection. Even for construct dGsw^{80}, only minor signals can be detected for helix P1 (U17, U77). Addition of Mg^{2+} and ligand folds dGsw^{78}–dGsw^{80} to the ligand bound state (Fig. 4b). Refolding to the ligand bound state is accompanied with an increase in the amount of imino proton signals, which appear as a result of tertiary interactions within the binding pocket (G24, G52, G75) and loops L2 and L3 (U66, G37, G38). In contrast, dGsw^{75}–dGsw^{77} maintain the identical imino proton pattern as observed in the absence of ligand and Mg^{2+}. These results are supported by analysis of the ligand proton imino signal intensity (Fig. 4c), which shows a sigmoidal dependence with increasing RNA length. In fact, the largest increase in ligand binding affinity can be observed when transitioning from dGsw^{77} to dGsw^{78}. While minor ligand binding can be detected for dGsw^{77}, the imino proton pattern does not show any conformational change towards the tightly arranged ligand-bound state. Therefore, the ligand is likely recognized by the aptamer domain but ligand-induced structural reorganization cannot take place due to insufficient stability of the P1 helix with only three base pairs. As a result, reorganization towards the ligand-bound state require a P1 of at least four base-pairs.

Conclusion

The work presented here combines and improves existing methods which now allow screening of a large number of different RNA constructs using NMR at single nucleotide resolution. We show that run-off transcriptions can yield highly homogeneous 3′ ends provided that transcriptions are performed in 20 % of DMSO and the transcription template is 2′-methoxy modified. This optimization allows a quick preparation of transcription templates for a multitude of RNAs on the basis of PCR by simple variations in the primers. On the downside, 5′ end ribozymes cannot be used and the RNA must be modified at the 5′ end to allow

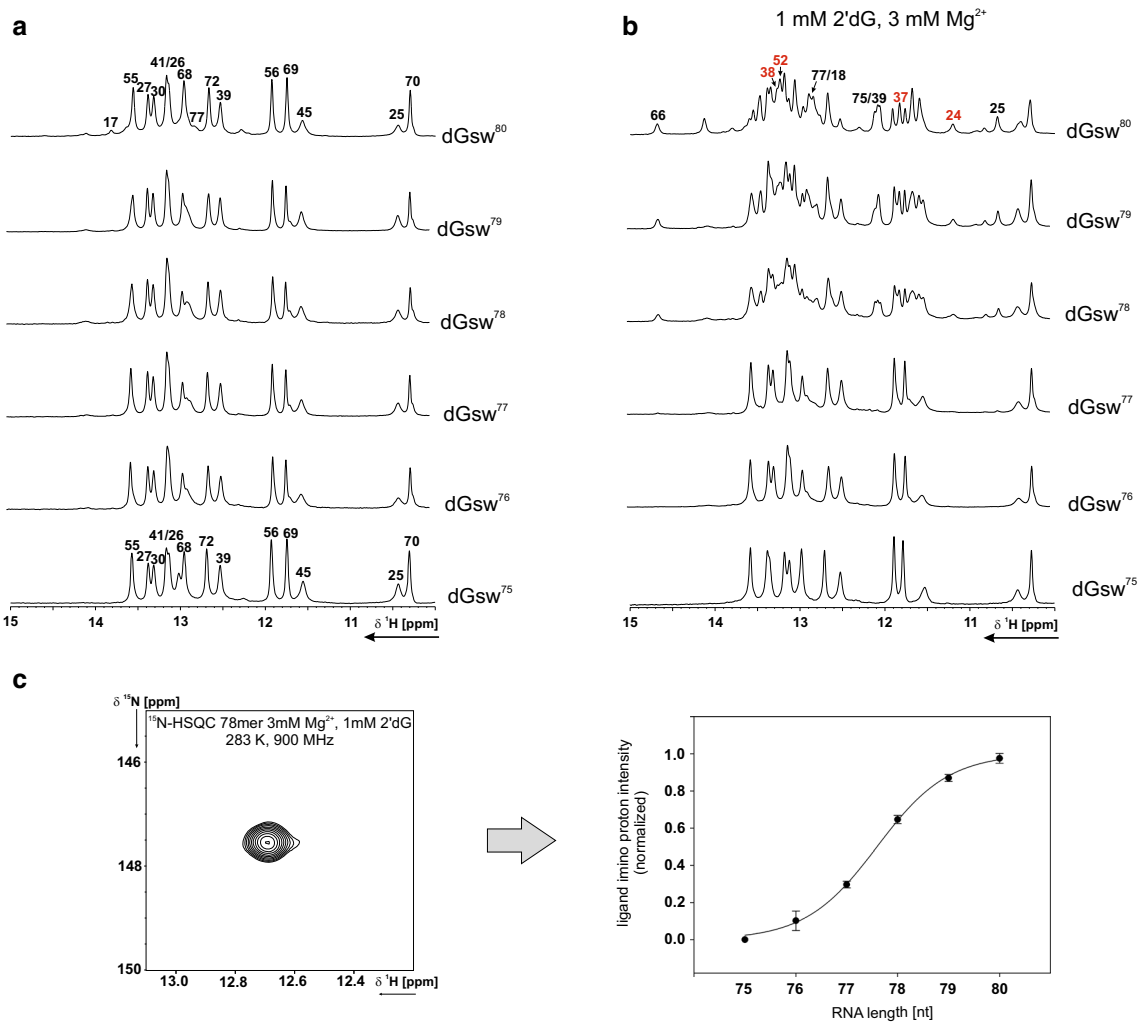

Fig. 4 a NMR screening of dGsw aptamer transcriptional intermediates. **a** ^{1}H-NMR spectra of buffer exchanged transcriptions. **b** ^{1}H NMR spectra of buffer exchanged transcriptions after addition of 3 mM Mg^{2+} and 1 mM of ^{15}N-labeled 2′dG. Signal assignments highlighted in *red* correspond to tertiary interactions and signals assigned in *black* correspond to stabilized closing base-pairs in the ligand bound state. The spectra were recorded at 298 K and 900 MHz. **c** Graphical representation of the imino proton signal intensity development with increasing RNA chain length determined by ^{15}N-HSQC spectra. The spectra were recorded at 283 K and 900 MHz. The signal intensity was normalized to a sigmoidal function fit: $Int = \frac{a}{1+e^{-(x-x_0)/b}}$ with a = 1, $x_0 = 77.6$, b = 0.7, $R^2 = 0.9989$. *Error bars* correspond to the standard deviation of the noise

transcription initiation by T7 RNA polymerase. Therefore, it needs to be verified that 5′ end design ensures correct folding. We show that buffer exchange and concentration of the transcription mixture yields near to identical NMR secondary structure imprints compared to PAGE- or HPLC-purified RNA samples. These findings allow rapid NMR analysis, which could potentially be applied to secondary structure screening of RNA mutations or screening of mRNA folding landscapes. We show the applicability of the screening procedure by monitoring the change in ligand affinity with increasing RNA chain length for a riboswitch aptamer domain. While during the time of measurements, the RNA has most likely reached its thermodynamic equilibrium, the method could nevertheless reveal possible misfolding pathways of kinetically stabilized structures occurring in non-coding mRNA during transcriptional elongation.

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