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Analysis of RNA Folding by Native Polyacrylamide Gel Electrophoresis

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Abstract

Polyacrylamide gel electrophoresis under native conditions (native PAGE) is a well-established and versatile method for probing nucleic acid conformation and nucleic acid-protein interactions. Native PAGE has been used to measure RNA folding equilibria and kinetics under a wide variety of conditions. Advantages of this method are its adaptability, the ease of radiolabeling RNA, an absolute determination of reaction endpoints, and direct analysis of conformational heterogeneity within a sample. Native PAGE is also useful for resolving ligand-induced structural changes.

1. Introduction

Polyacrylamide electrophoresis under non-denaturing conditions (native PAGE) has become a popular method for analyzing nucleic acid-protein complexes (Fried and Crothers, 1981; 1981), DNA bending and flexibility (Wu and Crothers, 1984; Koo et al., 1986) and conformational changes in RNA (Stahl et al., 1979; Pyle et al., 1990; Emerick and Woodson, 1994; Friederich and Hagerman, 1997). One source of its popularity is its adaptability: native PAGE can be used over a wide range of conditions to measure folding reactions, ligand binding, and even to select populations for in vitro evolution (Tuerk and Gold, 1990; Bevilacqua and Bevilacqua, 1998; Kim et al., 2003; Ryder et al., 2008). The permutations on this method are limited only by the imagination of the experimenter. Another advantage is that native PAGE requires small amounts of material that can be produced with standard molecular biology techniques and very little specialized equipment.

Native PAGE offers two other advantages for quantitative studies of macromolecule interactions. First, different conformations of the macromolecule can be visualized and enumerated, as long as they have different electrophoretic mobilities. This is also an advantage when measuring RNA-protein binding, as complexes with different stoichiometries can be distinguished (Fried and Daugherty, 1998). Second, the fraction of the population in each conformational state can be quantified in absolute terms. The latter is enormously helpful in obtaining reliable end-points for folding or binding reactions. In

contrast, footprinting data and spectroscopic data such as FRET must often be interpreted relative to some saturation point, with the assumption that all of the components in the system are active.

Native PAGE has several disadvantages. First, it is pseudoequilibrium method, like nitrocellulose filtration and footprinting. The separation of folded or bound polynucleotide requires that macromolecules are “caged” within the gel matrix during several hours of electrophoresis (Fried and Liu, 1994). As discussed below, the quality of the separation and interpretation of the results depends on the dynamics of the system and the rate of transport through the gel. Second, the electrophoretic mobility cannot be easily predicted from theory and thus interpreted in terms of a physical property such as hydrodynamic radius. For these reasons, it is desirable to complement native PAGE studies with other methods such as activity assays, footprinting, UV and fluorescence spectroscopy or small angle scattering.

This chapter will focus on the use of native PAGE to investigate folding of the *Tetrahymena* group I ribozyme. However, these protocols are easily adapted to other ribozymes and structured RNAs (e.g. (Pinard et al., 2001; Lafontaine et al., 2002; Adilakshmi et al., 2005; Severcan et al., 2009)). Detailed discussions of gel mobility shift methods for measuring protein-nucleic acid interactions are available elsewhere (Fried and Daugherty, 1998; Ryder et al., 2008). Below we discuss the parameters that govern the design of a native PAGE experiment and present protocols for the analysis of RNA folding reactions.

2. Theory of gel electrophoresis

2.1 Mobility of macromolecules

There are a number of excellent reviews of the theory of electrophoresis in free solution and in gels (Zimm and Levene, 1992; Sartori et al., 2003); for a synopsis of reptation theory see (Bloomfield et al., 2000). The velocity v of a charged particle in a solution with an electric field E depends on the electrical force $F_{el} = ZqE$, in which Z is the number of charges, q is the charge of a proton, and the frictional force $F_{fr} = -fv$, in which f is the frictional coefficient. At steady-state, these forces balance and the velocity is $v = ZqE/f$. The electrophoretic mobility μ is the velocity relative to the field strength, $\mu = v/E = Zq/f$.

During gel electrophoresis, the migration of macromolecules is obstructed by the polymer matrix, and thus depends on the molecular weight as well as the frictional coefficient with the matrix. In the reptation model, DNA is assumed to move in a worm-like fashion through virtual tubes in the gel polymer matrix (Figure 1a). This assumption is supported by direction observations of stretched DNA molecules (Perkins et al., 1994; Kantor et al., 1999). The central result is that the electrophoretic mobility depends on the mean-square end-to-end distance of the macromolecule, and inversely on length (L) or molecular weight. The electrophoretic mobility is expressed as

$$\mu = \frac{\langle h_x^2 \rangle Z_q}{L^2 f_{gel}} \quad (1)$$

in which L is the contour length (related to the number of residues) and h_x is the component of the end-to-end vector of the polymer that is aligned with the electric field (Zimm and Levene, 1992). Thus, DNA molecules that are bent have a shorter end-to-end distance and migrate more slowly than those that are straight (Wu and Crothers, 1984).

Another prediction of reptation theory is that molecules move fastest when the entire chain is in the same tube. Partial unfolding or branching of the helix make this less likely, and consequently impede migration, resulting in anomalous migration patterns that can be used to model helical junctions and bend angles (Zinkel and Crothers, 1990; Lilley, 2008). In practice, the migration of a nucleic acid through the gel is complicated by its interactions with the ions in the gel running buffer (Mohanty and McLaughlin, 2001; Stellwagen et al., 2003), field-dependent bias in the orientation of the leading edge of the DNA, and by the elasticity of the gel matrix (Zimm and Levene, 1992).

Folded RNAs are similar in size to the pores of a typical 4–20% polyacrylamide gel (1–8 nm) (Chrambach and Rodbard, 1971) and thus may be more appropriately described by a sieving model in which the macromolecule must avoid collisions with the gel matrix (Sartori et al., 2003) (Figure 1b). In this model, proposed by Ogston (Ogston), the gel mobility μ relative to the mobility in free solution μ_0 decreases with gel concentration c and a retardation coefficient K_R , or $\log \mu = \log \mu_0 - K_R c$ (Ferguson, 1964). Therefore, tightly folded RNAs travel more rapidly than unfolded RNAs of the same molecular weight. The observed electrophoretic mobility is difficult to predict, however, because of dynamic fluctuations in the macromolecule and in the gel matrix itself (Locke and Trinh, 1999; Stellwagen et al., 2003; Yuan et al., 2006). Happily, observation of an empirical change in gel mobility is sufficient for many applications.

2.2 Chemical exchange

The dynamics of the system are an important consideration when native PAGE is used to analyze RNA folding or protein-nucleic acid interactions. If chemical exchange between conformers (or bound and free RNA) is rapid relative to the rate of transport through the gel, a single band of average mobility will be observed (Cann, 1996). If exchange is slow relative to the rate of transport, then bands with different mobilities will be observed, corresponding to each conformational state. If the exchange rate is comparable to the rate of transport, then an additional band or zone of intermediate mobility will also be observed, representing material that has undergone exchange during electrophoresis. Even a small amount of exchange can result in fuzzy bands or a faint track of labeled material between the two principle bands.

Examples of RNA conformers in fast exchange can be found in folding studies on the P4-P6 domain of the *Tetrahymena* ribozyme and the yeast bI5 mitochondrial ribozyme (Szewczak and Cech, 1997; Buchmueller et al., 2000). In these studies, the average electrophoretic mobility increased as more of the RNA is folded, but only a single band was observed (Figure 2a). The fraction of folded RNA is extracted from the mobility of the RNA, relative to some standard such as a DNA restriction fragment or relative to the maximum mobility of the RNA when it assumed to be completely folded. The mobility of the P4-P6 RNA

decreased with the gel percentage, further confirming that mobility reflects the average compactness of the RNA (Figure 2b) (Szewczak and Cech, 1997).

Studies on the *Tetrahymena* ribozyme and pre-RNA provide an example of RNA conformers in slow exchange (Emerick and Woodson, 1994; Pan et al., 2000). In this case, each conformer migrates at a characteristic rate, producing several distinct bands or if there are many structures, a broad smear of material (Figure 3a,b). The native RNA migrates fastest, while unfolded or misfolded forms migrate more slowly. Each band is quantified separately, and the fraction of folded RNA is obtained from the amount of material in the native band. In general, we have found that even small changes in electrophoretic mobility reliably signal a change in structure that ought not to be ignored.

The ability to separate liganded forms of an RNA by native PAGE depends not only on the rate of chemical exchange, but also on the size of the molecule and its rate of diffusion within the pores of the gel during electrophoresis. In general, complexes with small molecules are more difficult to detect by native PAGE than complexes with large molecules such as proteins. Moreover, positively charged ligands move opposite the RNA in an electric field. Thus, ions such as Mg^{2+} must be added to the running buffer so that the RNA remains associated with Mg^{2+} ions as it travels through the gel. Interactions between lambda N peptides and box B RNA hairpins were successfully measured by adding peptide to the gel before polymerization (Cilley and Williamson, 1997).

3. Electrophoresis equipment

A variety of commercial apparatuses or “gel boxes” for vertical PAGE are suitable for native PAGE experiments. Temperature control is critical to the separation of RNA conformers (slow exchange), and thus the box must be designed for use with a circulating water bath. We have obtained good results with an Owl Penguin P10DS gel box (ThermoScientific) connected to a refrigerated recirculating water bath. Cold water circulates from the water bath through a central core that lies between two vertical polyacrylamide gel sandwiches. The surfaces that contact the gel sandwich are made of alumina, in order to improve heat transfer from the gel. A 20×20 cm format supplies the most area for critical separations, but smaller formats may be satisfactory for some applications. In addition to casting materials (plates, spacers, combs), an electrophoresis power supply capable of delivering 1000 V and maintaining constant power is also needed. It is helpful to place a small water bath or temperature block nearby for incubating samples prior to loading.

In general, we find that the internal temperature of the gel must be 10°C for good separation of RNA conformers. Separation of the *Tetrahymena* ribozyme is lost when the temperature rises above 15°C . With our apparatus, an internal temperature of 10°C can be achieved by setting the water bath to $0\text{--}4^\circ\text{C}$ and by applying no more than 15 W electrical power to each gel (or 30 W for two gels). The water bath should be filled with 1:1 (v/v) water:ethylene glycol to prevent icing. We insulate the tubing from the water bath with foam to help maintain a low temperature. If the temperature of the water bath is set below 2°C , care should be taken that ice or precipitates in the running buffer do not obstruct the current and produce uneven results.

The water bath must recirculate vigorously and have adequate capacity. Although the running buffer may be 2–4 °C, the gel interior is warmer owing its electrical resistance. We use an old Neslab RTE-110 (5 L) with cooling capacity of 300 W at 0 °C and 12 L/min pumping rate, which can dissipate the heat produced by 15 W electrical power per gel. This apparatus can cool up to two double-sided gel boxes. Newer refrigerants are less efficient, and may require higher capacity or lower electrophoresis power. Running gels in a cold room does not provide sufficient heat transfer and is unsuitable for the analysis of RNA conformers (although it may work for protein gel mobility shifts).

4. Stability of folded RNA measured by native PAGE

4.1 Casting and pre-running gels

Solutions for native polyacrylamide gels are prepared and cast in glass frames according to standard methods. We use 0.5 mm thick teflon spacers and combs with 24 to 30 wells in a 20 cm-wide gel. Short and long glass plates are assembled with side spacers and clamped on each side with large black binder clips. Polymerization of native gels is particularly sensitive to contaminants. Grease or silicone lubricants are to be avoided and plates should be cleaned meticulously with soap, water and finally ethanol before and after each use. The bottom of the casting frame may be sealed with electrical tape if desired, although this is not necessary.

To resolve folded and unfolded conformations of the 387 nt *Tetrahymena* ribozyme, we use 8% acrylamide (29:1 mono:bisacrylamide) in 34 mM Tris, 66 mM Hepes (pH 7.5), 0.1 mM EDTA, and 3 mM MgCl₂ (THEM₃). Hepes is used instead of borate to maintain the native structure of the ribozyme (Pyle et al., 1990; Buchmueller and Weeks, 2004), while the MgCl₂ concentration is chosen to be just sufficient to maintain the RNA in its folded state. Prepare and degas 25 ml of acrylamide solution per gel using RNase-free water. Add 200 µL 10% ammonium persulfate and 50 µL TEMED to begin polymerization, and pour immediately into the prepared frame. Insert the comb and lay the gel frame flat until the polyacrylamide has polymerized completely (about 45 min).

When the gel has polymerized, remove the comb and flush wells thoroughly with deionized (RNase-free) water. At this stage, the gel can be covered with plastic wrap and stored overnight at 4 °C if desired. Remove binder clamps (and tape if used) and place the gel on the gel box. Fill the upper and lower reservoirs with 1X THEM and pre-run for 30–45 min with the water bath at the desired temperature, making sure that the current is not blocked by ice, precipitate or air bubbles. The gel temperature can be probed with a contact thermometer or small thermal probe inside the gel.

4.2 Sample preparation

RNA samples for native PAGE can be prepared in many different buffers or salts and at different temperatures, depending on the scientific question to be addressed. We provide a protocol for measuring the folding of the *Tetrahymena* ribozyme that can be adapted to other RNAs as needed. Since native gels are easily overloaded, best results are obtained with low (0.1–10 µg/mL) RNA concentrations, although we have used up to 1 mg/mL RNA. Sample volumes should be 2–4 µL per lane to produce tight bands. To detect small quantities of

RNA, the RNA must be labeled with a fluorescent dye or a radioisotope. ^{32}P -labeling is most sensitive and easily quantified with storage phosphorescence scanners.

To measure the folding equilibrium of the *Tetrahymena* L-21Sca ribozyme in various counterions, ^{32}P -labeled ribozyme is prepared by T7 in vitro transcription with α -ATP, according to established protocols (Zaug et al., 1988). Free label is removed with a size-exclusion spin column (eg, TE-10, BD Sciences), and the RNA used without further purification (Emerick and Woodson, 1993). If desired, the RNA can be end-labeled with ^{32}P , and purified by denaturing PAGE. As for all biochemical experiments on RNA, care must be taken to use water, reagents and plastic consummables that are free of RNase.

For the folding reactions (5–10 μL), labeled ribozyme (1,000–2,000 cpm/ μL) is added to HE buffer (50 mM Hepes adjusted to pH 7.5 with sodium hydroxide, 1 mM EDTA pH 8), 10% (v/v) glycerol, 0.1% (v/v) xylene cyanol, plus the desired concentration of MgCl_2 or other salt (Heilman-Miller et al., 2001; Koculi et al., 2004). At least one sample should contain no MgCl_2 , representing the “unfolded” RNA, and one sample should contain enough MgCl_2 to fold the RNA completely. The reactions are incubated at the desired temperature for sufficient time for the reaction to reach equilibrium. We incubate the *Tetrahymena* ribozyme 2 to 4 hours in a water bath at 30°C. Shorter incubation times suffice at higher temperatures, or for RNAs that fold rapidly (Rangan et al., 2004).

4.3 Running the gel

When the folding reactions have reached equilibrium, 2 μL of each sample are loaded into separate lanes of a native 8% polyacrylamide gel that was prepared and pre-run as described above (<10°C).^{9,14,25} It is helpful to use narrow tips that allow the sample to be placed near the bottom of the well. The current should be applied to the gel as soon as possible after the samples are loaded. Gels must be run long enough to resolve the conformational species of interest, but not so long that smaller RNAs run off the bottom of the gel. To resolve the folded and unfolded forms of the *Tetrahymena* ribozyme, gels are run 4 h at 15 W.

In this protocol, glycerol and tracking dyes were added to the RNA folding reaction, so the samples can be loaded directly without further manipulation. However, glycerol slightly perturbs the folding free energy of the ribozyme (Pan et al., 1999), and prevents the formation of some RNA-protein complexes (Bellur and Woodson, 2009). An alternative approach is to prepare samples without glycerol, then rapidly mix it with a tenth volume of glycerol and dyes just before loading aliquots on the gel.

At the end of the run, drain the buffer reservoirs and disassemble the gel frame, taking appropriate precautions if using radioactive samples. Carefully remove one glass plate and transfer the gel to a piece of Whatman 3mm filter paper. Cover the other side of the gel with plastic wrap and dry completely in a vacuum gel dryer at low heat (to avoid cracking). The dried gel is directly exposed to an imager or to X-ray film.

4.4 Data analysis

Although a qualitative analysis is sometimes sufficient, in most cases it is desirable to quantify the amount of labeled material in each conformational state. This can be done using

commercial or free image analysis software to integrate pixels within a certain area. One approach is to obtain an intensity profile of each lane and integrate the area under each peak (usually from one row of pixels, although some programs can average several rows (Das et al., 2005)). Area integration can distinguish broad and sharp peaks, and overlapping peaks can be sometimes deconvoluted. A disadvantage of this method is that the results are sensitive to distortions in the gel because only a thin strip of each lane is used.

Alternatively, peaks areas can be defined manually and the entire volume of the peak integrated. This method is more tolerant of imperfect gels. However, because the bands in native gels are often broad, it is critical to set a uniform criterion (such as pixel intensity) for defining peak boundaries and to carefully subtract the background. One way to estimate the background is to integrate a similar number of pixels from a region of the gel in which no sample was loaded. Ideally, at least the folded RNA will produce a sharp band that can be clearly distinguished from other bands on the gel (Figure 3). If this is not the case, it may be worth optimizing the electrophoresis conditions to improve the separation or changing the folding buffer to increase the stability of the RNA.

Once the peaks have been integrated, the amount of each labeled species relative to the total is easily calculated for each lane in the gel ($f_i = \text{counts}_i / \text{counts}_{\text{total}} \approx \text{counts}_i / \sum \text{counts}_j$). The results of the experiment can be fit to an appropriate model. For folding experiments, the fraction of native RNA versus Mg^{2+} concentration C was fit to the Hill equation,

$$f_N = f_N(0) + [f_N(\text{max}) - f_N(0)] \left[\frac{(C/C_m)^n}{1 + (C/C_m)^n} \right] \quad (2)$$

where $f_N(0)$ is the fraction of folded RNA without Mg^{2+} , $f_N(\text{max})$ is the maximum fraction of folded RNA in saturating Mg^{2+} , C_m is the midpoint of the folding transition and n represents the cooperativity of the folding equilibrium with respect to Mg^{2+} (Figure 3c). Alternatively, the free energy of the folding transition can be calculated from

$$\Omega = \frac{1}{8} \left(\frac{\Delta G_{UN}}{RT} \right)^2 \frac{1}{\ln(3 + 2\sqrt{2})} = \frac{C_{\text{max}}^2 (df_N/dC)_{\text{max}}}{\Delta C} \quad (3)$$

where R is the gas constant, T is temperature, and ΔG_{UN} is the free energy change associated with the Mg^{2+} -dependent folding transition (Pan et al., 1999). Ω is a dimensionless quantity that is related to the Hill coefficient and the midpoint of folding transition, C_{max} is the concentration of Mg^{2+} at which the derivative of fraction native with respect to C reaches its maximum, $(df_N/dC)_{\text{max}}$ is the maximum value of the derivative, ΔC is the width of the curve at $1/2(df_N/dC)_{\text{max}}$. This method has the advantage of being less sensitive to variations in the upper and lower baselines of the curve. In both cases, ΔG_{UN} is assumed to vary with $\ln C$, so that $\Delta G_{UN} = \Delta G_{\text{ref}} - n \ln C$ (Fang et al., 1999; Pan et al., 1999).

5. RNA folding kinetics

Native PAGE can also be used to measure the kinetics of RNA folding. The time resolution of the method is limited by the time needed to mix the samples and to load them into the well of the gel itself, which typically require 15–30 s. Thus, this method is only suitable for reactions with half-lives greater than 1 min (Fried and Crothers, 1981). As discussed below, the folding reaction effectively stops once the samples enter the gel, trapping the molecules in whatever conformation they held when they entered the gel. Refolding of the *Tetrahymena* ribozyme is very slow at the low temperatures of the running buffer and the gel.

Because samples must be loaded on the gel immediately, the native gel is cast and pre-run before the start of the reaction. Folding reactions are set up in a sufficient volume so that aliquots can be withdrawn at various times (20–40 μ L). We obtain the most reproducible results by first mixing all of the reaction components except the RNA. This mixture is warmed to the desired reaction temperature (eg 30–50 $^{\circ}$ C) in a water bath or heating block placed near the native gel apparatus. The folding reaction is then started by adding a 5X or 10X stock of unfolded RNA to the folding buffer.

To follow the reaction over time, a 2 μ L aliquot is removed as soon as possible (eg 15 s) and loaded in the first well of the gel. With the current running, reaction aliquots are loaded in adjacent wells at different times, with intervals chosen to span the expected half-life of the reaction. It is recommended that one also prepare samples of unfolded and fully renatured RNA as controls.

The gels are run and analyzed as described above. The increase in the fraction folded RNA over time is fit to a rate equation, such as

$$f_N(t) = A_{\max}[1 - \exp(-kt)] \quad (4)$$

in which k is the observed rate constant and $A_{\max} \approx f_N(\infty)$ is the maximum amplitude of the reaction. One difference between time courses and equilibrium experiments is that samples loaded at the beginning of the reaction travel further through the gel than samples loaded at the end of the reaction (Figure 3b). The resulting curvature in the banding pattern does not interfere with the analysis as long as all lanes run long enough to achieve the desired resolution of the sample. Control samples can be loaded at the beginning and the end of the time course to facilitate band assignment. If the experiment is performed correctly, the endpoint of the progress curve should agree with the fraction of folded RNA in equilibrium experiments under the same condition.

6. RNA compactness and native PAGE mobility

As discussed above, the ability of folded RNAs to migrate through the gel depends on their size relative to the pores of the gel and on structural fluctuations that “snag” the RNA on obstacles within the matrix during electrophoresis. This principle can be used to estimate the

compactness of the folded RNA, as more tightly folded RNAs should sieve through the gel more easily.

Buchmueller and Weeks (Buchmueller et al., 2000) measured the mobility of the bI5 ribozyme in native gels containing 0 to 20 mM MgCl_2 . Double-stranded 174 bp RNA, which is not expected to change structure upon the addition of Mg^{2+} , was loaded on the same gel as a control. The velocity of the ribozyme through the gel relative to the dsRNA control increased with Mg^{2+} , indicating the RNA was more tightly folded. This change in mobility correlated with a decrease in the Stokes' radii (R_H) measured by gel permeation chromatography (Buchmueller et al., 2000).

We measured the mobility of the *Tetrahymena* ribozyme relative to DNA restriction fragments in native polyacrylamide gels containing MgCl_2 , CaCl_2 and SrCl_2 (Koculi et al., 2007). In these studies, the folded RNA migrated fastest in MgCl_2 and slowest in SrCl_2 , consistent with a less tightly packed and more dynamic structure in the larger cations that was also reflected in the breadth of the RNA peak in gel permeation chromatography (Koculi et al., 2007) (Figure 4). We also measured the radius of gyration (R_g) of the *Tetrahymena* ribozyme by small angle X-ray scattering, and found that it differs very little in MgCl_2 , CaCl_2 and SrCl_2 (G. Caliskan and S.A.W., unpublished data). Thus, the data from native PAGE must be interpreted cautiously, as both size and dynamic fluctuations can lower the mobility of an RNA (Olson et al., 1993).

7. Probing the function of conformers resolved by native PAGE

One of the most important questions is how the conformational states resolved by native PAGE differ in their structure and function. Sometimes the bands can be assigned to functional states by comparing the results with those from other assays. For example, the proportion of the fast migrating band of the *Tetrahymena* pre-RNA at different MgCl_2 concentrations correlated with its self-splicing activity under the same conditions, allowing this band to be assigned to the native state (Pan and Woodson, 1998). This correlation between solution activity and native PAGE results also provided reassurance that the amount of folded RNA trapped by the native gel corresponded faithfully with the proportion of active RNA.

7.1 Measuring RNA activity *in situ* with two-dimensional PAGE.

An important control is to determine whether the species with different electrophoretic mobility can exchange. This is done by eluting RNA from each band in the native gel, incubating it for an appropriate time under folding conditions, and repeating the native PAGE analysis. Assuming the RNA conformations are in exchange, it is not possible to determine the activity of each species in solution. However, the reactivity or conformation the RNA can be probed *in situ* by diffusing substrates or modifying reagents into the gel. Detailed protocols for “fingerprinting” RNA conformers can be found elsewhere (Woodson, 2001).

For the *Tetrahymena* pre-RNA, native and partly misfolded conformers were first separated on a 6% native gel as described above (Emerick and Woodson, 1994). After the first round

of electrophoresis, gel slices corresponding to each lane were excised and laid on the bottom of a glass plate for a second round of denaturing electrophoresis. A solution of GTP, which is a co-factor for self-splicing of the pre-RNA, was pipetted onto the surface of the gel slices. After a few minutes, self-splicing was quenched by applying a solution of 8M urea and EDTA. A second plate and spacers were added to the first plates, and the denaturing polyacrylamide gel cast in place over the gel slices from the first dimension. The second dimension of denaturing PAGE resolved the products of the self-splicing reaction, which are lower in molecular weight. Only the fast migrating band gave rise to fully spliced RNAs, demonstrating that this band represented the native (N) form of the pre-RNA (Emerick and Woodson, 1994).

An alternative approach is to probe the conformation of the RNA in the native gel by chemical modification (Emerick and Woodson, 1994). Rather than use two-dimensional electrophoresis, each band was excised from the native gel and placed in a 1.5 mL tube on ice. A solution of dimethylsulfate (DMS) was pipetted over the gel slice, followed by a quench of 1 M beta-mercaptoethanol. The modified RNA was then eluted from the gel slice and analyzed by primer extension (Woodson, 2001). This approach has the advantage of probing the RNA structure *in situ*. However, the extent of modification within the gel slice is difficult to control, and many chemical modification reagents are not reactive enough to work within the gel matrix. An alternative is chemical modification interference or nucleotide analog interference (NAIM) (Christian and Yarus, 1992; Ortoleva-Donnelly et al., 1998; Pan and Woodson, 1998). In these methods, the RNA is modified in solution before native PAGE, to identify modifications that interfere with folding or ligand binding.

7.2 Ligand-induced conformational change

Another method of probing the function of and RNA's conformational states is to determine whether each conformational species can interact specifically with ligands. If the ligand changes the mobility of the RNA, then ligand binding can be assessed simultaneously by native PAGE. For example, Draper and co-workers separated two isoforms of a pseudoknot within the *E. coli* alpha operon mRNA by native PAGE (Gluick et al., 1997). Ribosomal protein S4, which represses translation of the alpha operon, selectively retarded the migration of the fast isomer, showing that the fast form of the RNA is bound by the protein (Schlax et al., 2001).

We observed that GTP shifts the *Tetrahymena* pre-RNA to a conformation that migrates just a bit more slowly than the native pre-RNA (Emerick et al., 1996; Pan et al., 1999). Only the native form of the RNA was affected; the mobility of bands containing unfolded or misfolded RNA did not change in the presence of GTP. The shifted band contains a complex of spliced RNAs (Emerick et al., 1996), and was thus a useful diagnostic for the RNA's catalytic activity (Pan et al., 1999).

8. Controls and further considerations

Because conformational changes in RNA or short DNAs typically cause small changes in electrophoretic mobility, analysis of nucleic acid folding requires careful optimization of electrophoresis conditions. By contrast, protein-nucleic acid interactions are typically easier

to analyze by native PAGE because the molecular weight and positive charge of the protein produces a relatively large shift in gel mobility.

In designing the experiments, the native conformation of the RNA (or the RNA-protein complex in a gel shift assay) must be trapped in the matrix of the gel during loading of the sample and remain stable during the electrophoresis run. First, the concentration of MgCl_2 in the running buffer, as well as the buffer itself, can be varied, depending on the stability of the RNA to be studied. Second, the polyacrylamide concentration and crosslinker ratio should be optimized for each system. We have used 6% polyacrylamide (29:1 mono:bis) for a 500–700 nt RNAs, 8% for 200–400 nt ribozymes and 8–12% for oligonucleotides.

Evidence that native PAGE results reflect solution conditions rather than conditions in the gel come from experiments on the *Tetrahymena* pre-RNA in different ions. While the *Tetrahymena* ribozyme can fold in Ca^{2+} , splicing requires Mg^{2+} ions in the active site (Grosshans and Cech, 1989; Streicher et al., 1996). As discussed above, a GTP-dependent mobility shift is diagnostic for the catalytic competence of the pre-RNA (Pan et al., 1999). The GTP-dependent shift was observed when Mg^{2+} was added to the samples and Ca^{2+} was added to the gel running buffer, but not when the samples contained Ca^{2+} and the gel contained Mg^{2+} . Thus, the native PAGE results reflect the conformational state of the RNA before it was loaded on the gel, rather than any changes that might have occurred in the gel itself.

A final concern is the extent to which the proportion of each conformer is faithfully captured by the native gel. Although very small structural differences, such as isomerization within an active site, may not be resolved unless they alter the hydrodynamic profile of the RNA, native PAGE results generally correlate well with other measures of RNA folding in solution. Because 10–30 s are needed for samples to enter the gel, this native PAGE is most successful in resolving conformational states that do not exchange within this time (Figure 3a). The entrapment of different conformers is aided by the low temperature of the running buffer, which slows conformational exchange in the sample well and in the gel itself.

For example, misfolded forms of the *Tetrahymena* ribozyme refold very slowly at 4 °C, and are easily separated from the native form (Pan and Woodson, 1998). However, if the ribozyme is first incubated in another ion such as Na^+ that allows the RNA to come close to the native structure, these native-like intermediates are captured as the native form when the RNA encounters Mg^{2+} in the gel running buffer (Heilman-Miller et al., 2001). Similarly, the *Azoarcus* ribozyme rapidly forms native-like, compact intermediates in Mg^{2+} concentrations below that required for catalytic activity (Rangan et al., 2003). These intermediates also appear in the folded state when assayed by native PAGE.

9. Summary

Native PAGE is a versatile method for probing the equilibria and kinetics of RNA folding reactions, and the interactions between RNAs and their ligands. Its principle advantage is the ability to resolve and quantify conformational heterogeneity within a system. Native PAGE is best suited for resolving large scale structural changes and those that are in slow

exchange. The mobility of individual macromolecules is also sensitive to conformational fluctuations during electrophoresis, and future developments may lead to further use of electrophoresis through gels and soluble polymers for the study of molecular dynamics (Sartori et al., 2003).

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Figure 1. Models for macromolecular electrophoresis. (a) Reptation of long DNA fragments in an electric field. Redrawn from (Bloomfield et al., 2000). (b) Ogston sieve model, which applies when R_g of the macromolecule is smaller than the diameter of the pore. Counterions move in the opposite direction of the RNA. (c) Scanning electron micrograph of 7.5% polyacrylamide gel. Reprinted from (Yuan et al., 2006).

Figure 2.

Native gel electrophoresis of the *Tetrahymena* P4-P6 RNA. (A) The folded and extended forms equilibrate rapidly, producing a single band whose mobility reflects the average structure of the RNA. U1, U2, BP5/5a refer to mutations in a hinge region. RNAs were run on native 6% (19:1) polyacrylamide gel in TBE + 10 mM MgCl_2 at 25 °C. (B) Ferguson plot shows that the relative mobility depends linearly on gel concentration, as predicted by the Ogston model. Reprinted from (Szewczak and Cech, 1997).

Figure 3.

Native gel electrophoresis of the *Tetrahymena* ribozyme. (A) Folded (N) and misfolded (I) forms of the RNA are in slow exchange and are trapped within the matrix of the gel, migrating at different rates. Folded RNA is stabilized by Mg^{2+} in the running buffer. (B) Folding kinetics. Ribozyme was incubated in folding buffer containing $MgCl_2$ for 0–10 min before samples were loaded on a native 8% (29:1) polyacrylamide gel in $THEM_3$ at 4 °C. wt, wild type; L2P5cP3 is a mutant that increases the folding rate. The fraction of native RNA is determined from the volume of counts in the native band relative to the total counts in the lane. (C) Folding equilibrium of the ribozyme in different ions measured by native PAGE. The fraction of N was fit to the Hill equation. Redrawn from (Pan et al., 2000) and (Heilman-Miller et al., 2001).

Figure 4.

Mobility of the *Tetrahymena* ribozyme in different divalent metal ions. (A) The unfolded (U) and folded (F) ribozyme was run next to Φ X DNA size markers on native 8% PAGE in THE buffer with 3 mM MgCl₂, CaCl₂ or BaCl₂. (B) The relative RNA mobility decreased with the size and charge density of the metal ion. Reprinted from (Koculi et al., 2007).