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Use of Biotin-Labeled Nucleic Acids for Protein Purification and Agarose-Based Chemiluminescent Electromobility Shift Assays

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We have employed biotin-labeled RNA to serve two functions. In one, the biotin tethers the RNA to streptavidin–agarose beads, creating an affinity resin for protein purification. In the other, the biotin functions as a label for use in a modified chemiluminescent electromobility shift assay (EMSA), a technique used to detect the formation of protein–RNA complexes. The EMSA that we describe avoids the use not only of radioactivity but also of neurotoxic acrylamide by using agarose as the gel matrix in which the free nucleic acid is separated from protein–nucleic acid complexes. After separation of free from complexed RNA in agarose, the RNA is electroblotted to positively charged nylon. The biotin-labeled RNA is readily bound by a streptavidin–alkaline phosphatase conjugate, allowing for very sensitive chemiluminescent detection (~0.1–1.0 fmol limit). Using our system, we were able to purify both known iron-responsive proteins (IRPs) from rat liver and assess their binding affinity to RNA containing the iron-responsive element (IRE) using the same batch of biotinylated RNA. We show data indicating that agarose is especially useful for cases when large complexes are formed, although smaller complexes are even better resolved.
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The electromobility shift assay (EMSA,[2] also known as gel shift, bandshift, and gel retardation assays) has been in use to detect protein–nucleic acid interactions for about 20 years, and is still most often performed with very little modification to the original technique (1). Concerns about the neurotoxicity of acrylamide and radioactively labeled molecules led us to develop an alternative to the standard format. All EMSAs are presumed to provide a qualitative measure of the strength of protein–nucleic acid interactions unless quantitatively verified by a thermodynamically rigorous technique (2). Our choice to use biotin to label the RNA was further exploited to facilitate purification of the RNA-binding proteins we were attempting to isolate.

The use of radioactive methods for the labeling of nucleic acids is becoming largely supplanted by nonradioactive methods due to intrinsic health and safety concerns, short half-lives of some isotopes, fragmentation of nucleic acid chains that can occur in highly labeled samples, and costs of waste disposal. There are two main types of nonradioactive labeling systems currently in use. One is the use of intrinsic fluorescence or luminescence, and the second is the use of a secondary detection system which is fluorescent or chemiluminescent. Each method has merit for different applications.

Intrinsically fluorescent labels such as 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BIODIPY; Molecular Probes, Eugene, OR), rhodamine derivatives, or Big Dyes (Perkin–Elmer) are especially useful for automated tasks such as DNA sequencing (3, 4). Fluorescent probes can also be used for very sensitive detection of nucleic acids in gels (5). The utility of these techniques is limited only by the specificity of labeling and amplitude of the signal.

Other labels require secondary detection protocols (6). Popular examples of this type are digoxigenin (DIG) and biotin, which require anti-DIG antibody and streptavidin, respectively, to specifically recognize la-

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[2] Abbreviations used: IRP1, iron-responsive protein-1; IRE, iron-responsive element; EMSA, electromobility shift assay; DIG, digoxigenin; TBE, Tris–borate–EDTA.

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beled RNA, DNA, or protein molecules. These methods generally require that the labeled molecules be transferred to solid membrane supports such as positively charged nylon or nitrocellulose. One of the primary advantages of secondary detection methods is the signal amplification potential. For instance, one biotin group on an RNA molecule could be bound by one streptavidin molecule with multiple alkaline phosphatase molecules covalently attached. Each alkaline phosphatase can then carry out many reactions of a lumigenic substrate before "burning out," thus multiplying the original biotin signal into many secondary photons.

For the detection of RNA in gels, one of the most sensitive methods is the fluorescent dye SYBR Gold, which has a detection limit of ~100–500 pg/band (5). For comparison, the detection limit for the biotin–streptavidin–alkaline phosphatase system described herein is ~5–50 pg/band (0.1–1 fmol/band in terms of RNA chains). Beyond better sensitivity, one must also consider the likely possibility that protein–RNA complexes in gels may not stain as efficiently as free RNA. Therefore, we have elaborated on the use of other labeled nucleic acid systems to develop a less toxic and more expedient method (7, 8).

The replacement of acrylamide with agarose in our EMSA technique is also beneficial because it avoids the toxicity of acrylamide (and new governmental regulations requiring special handling procedures). Special high-resolution agarose has been used to successfully separate short DNA from protein/DNA complexes (11); however, we obtained excellent results with standard DNA-grade agarose. Agarose gels are also easier and cheaper to cast than polyacrylamide, and agarose is more amenable to resolving differences in the electrophoretic migration of large complexes due to its larger pore sizes (9). An excellent example of the resolving power of agarose-based EMSAs is the detection of the binding of the transcription factor Zta with seven operator sites spanning ~200 base pairs of radiolabeled DNA which would form a protein complex of ~420 kDa with DNA of about ~130 kDa (10). Inclusion of Mg^{2+} ions in the gel was required for proper complex resolution; however, the physical basis for this requirement is unknown.

Although safety is also relevant at larger research universities and biotechnology concerns, this technique is especially valuable for undergraduate student researchers, allowing them to perform EMSAs without the usual trepidation regarding radioactivity and acrylamide.

Another benefit of biotinylated RNA that we have discovered is that the same RNA used for EMSAs can be coupled to streptavidin-coated agarose beads. This method is similar in concept to affinity purification of DNA-binding proteins (12). The RNA is bound through the very strong noncovalent interactions of biotin with streptavidin. Proteins that specifically recognize an RNA structure can be bound to these RNAs in a quaternary complex (agarose–streptavidin–RNA–protein) and eluted with some suitable solution variable. We chose to use a step gradient of KCl; however, other salts, or pH, or other solution variables could, in theory, be used to elute the desired protein.

MATERIALS AND METHODS

RNA Synthesis and Labeling

Biotinylated RNA was synthesized by *in vitro* runoff transcription using T7 RNA polymerase (13) using a modified MaxiScript kit (Ambion, Austin, TX). A small amount of the rCTP was replaced with biotinylated rCTP containing an 11-carbon linker to achieve a labeling of approximately 4–6 biotin groups per 100 nucleotides (nt) of RNA (Roche Biochemicals, Indianapolis, IN). The DNA template corresponded to the mRNA of rabbit ferritin light chain. The RNA-labeled S-IRE (for "short IRE") is 150 nt long and contains the iron responsive element plus a small portion of the ferritin 5′ untranslated region and some polylinker region from the vector. The RNA-labeled L-IRE (for "long IRE") is 479 nt long and corresponds to the ferritin mRNA from the 5′ cap to approximately the middle of the open reading frame. The β-actin RNA is 304 nt long, and was transcribed from a commercial β-actin gene fragment (Ambion). The mRNAs of actin and ferritin have no homology. Alternatively, RNA can be synthesized *in vitro* without biotinylated precursors and then end-labeled with biotinylated rNTPs with RNA ligase.

The concentration of our biotinylated RNA was determined by interpolation from a standard RiboGreen fluorescence plot using known rRNA standards (Molecular Probes, Eugene, OR). The biotinylated RNA stocks were diluted by 30- to 100-fold with nuclease-free H_2O for use in the binding assays. The concentrated biotinylated RNA stocks are stable at −20°C for at least 2 years (data not shown).

Binding Buffers

The binding buffer for IRP1–IRE interactions was buffer A (10 mM KHepes, pH 7.4, 5% glycerol, 5 mM magnesium acetate, 40 mM KCl, 1.0 mM dithiothreitol, 67 ng/μl yeast tRNA, 1.5 μg/μl heparin) + 2% 2-mercaptoethanol. The tRNA and heparin are added as nonspecific competitors, and 2-mercaptoethanol is added to maintain IRP1 in the reduced, high-affinity RNA-binding state.

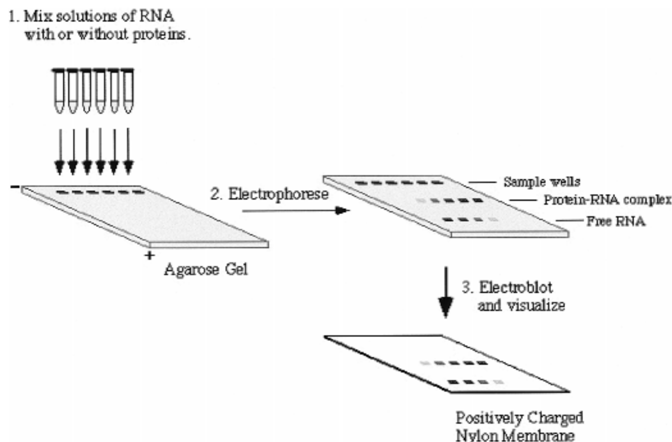

FIG. 1. Schematic representation of the EMSA protocol. Biotinylated nucleic acids are incubated with or without proteins and then applied to agarose gels and subjected to an electrophoretic field. The separated nucleic acids are electroblotted to positively charged nylon membrane and later detected via a streptavidin–alkaline phosphatase chemiluminescent detection system.

Electromobility Shift Protocol

Figure 1 shows a general overview of our EMSA technique. Briefly, biotin-labeled RNA or DNA is incubated in the presence or absence of proteins. All RNAs used in our EMSAs were heat-treated at 90–95°C for 3 min, followed by rapid cooling on ice just prior to incubation with proteins. This procedure was included to “melt” weak secondary structure and allow all of the RNA molecules to be present in the same conformation. Without this step, it was often found that free RNA ran as doublets or triplets (data not shown). Potential protein–RNA complexes are allowed to form (typically by incubating on ice for 20 min), and the solutions are transferred to wells in 8 × 10-cm submarine-style 1.2% agarose (standard LE grade) gels. The gels are subjected to electrophoretic fields in 0.5× Tris–borate–EDTA (TBE) buffer, pH 7.5, until separation has been achieved (typically 45–75 min at 100 V, depending on the size of the RNA). The RNA is then electroblotted at 200 mA for 20 min to positively charged nylon membrane (MagnaGraph, Micron Separations, Inc.) in a MiniProtean II electroblotter (Bio-Rad; Hercules, CA) using 1.0× TBE buffer, pH 8.3. (Capillary transfer also works; however, we chose electroblotting for its rapidity.) The RNA is crosslinked to the membrane by shortwave ultraviolet radiation for 3 min. Although transfer of the biotinylated RNA or DNA to positively charged nylon membrane is required, the whole process is still faster than standard EMSAs because of the very short exposure times needed to generate signal (see below). It routinely takes only 6–7 h from the initiation of binding reactions to the detection of signal, although weaker signals can be detected by longer exposure to X-ray film.

We have determined that, at least in the case of IRP1, the protein is not detectable by standard immunoprobing of the positively charged nylon membrane (data not shown). The protein could, in theory, be recovered on a nitrocellulose membrane placed behind the positively charged nylon membrane during the electroblotting. This could be useful to identify proteins in protein–nucleic acid complexes by means of immunoprobing, especially in cases where antibody “supershifts” are not feasible.

Detection of the RNA on the membrane is performed by washing the blot with a detergent solution, followed by a block solution (Brightstar Biodetect kit, Ambion). The membrane is then probed with streptavidin–alkaline phosphate conjugate to bind biotinylated RNA (Tropix; Bedford, MA). The blot is washed again, and substrate (CDP-Star, Tropix) is added to initiate the chemiluminescent reaction. The membrane is wrapped in plastic wrap to prevent drying of the membrane as well as to provide an optically translucent cover. The Saran-enshrouded membrane is exposed to X-ray film or photons are detected with a cooled CCD camera system for (typically) 10–30 min. We have determined that the light output from the blot is maximal at approximately 30–90 min post-substrate addition; however, considerable intensity is still evident at 24–36 h (data not shown).

Affinity Column Protocol

Covalently linked streptavidin–agarose beads (Sigma, St. Louis, MO) were slurried and placed in a fritted Bio-Rad Dispo-column fitted with a stopcock (Bio-Rad). The resin was washed exhaustively with buffer A to remove any unbound streptavidin. A solution of biotinylated S-IRE RNA was added to the resin, accompanied by reslurrying. This would produce a quaternary complex of agarose–streptavidin–biotin–RNA. Theoretically, any RNA- or DNA-binding protein could be purified by an analogous method as has been demonstrated (12).

Based on the manufacturer’s specifications for the resin that we used, the theoretical limit of binding RNA is 750–1500 μg of RNA per milliliter of resin, assuming that each RNA molecule binds to one streptavidin. If this level of RNA binding were obtained, it should be possible to bind as much as 1.5 to 3 mg of IRP1 per milliliter of resin. (Due to limitations in obtaining such quantities of biotinylated RNA, our column was loaded to less than 0.1% capacity with respect to RNA. Therefore, the maximal yield of IRP1 expected under our conditions was under 500 ng of IRP1. Because of this low yield, we were unable to

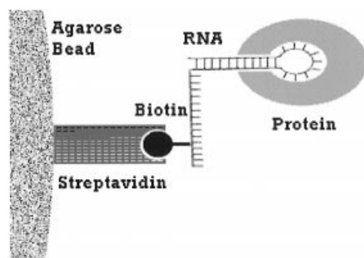

FIG. 2. Schematic representation of the affinity purification resin. Large agarose beads with covalently bound streptavidin bind biotin moieties on biotinylated RNA. The RNA serves as a noncovalent binding site for sequence/structure-specific RNA-binding proteins.

detect protein in denaturing gels to verify purity. However, based on EMSA binding activity, we calculate that our total yield was approximately 100 ng of IRP1.)

Once the resin settles, it is again washed exhaustively with buffer A. The solution containing the RNA-binding proteins (e.g., IRP1) is then added and slurried to achieve maximal binding. As depicted in Fig. 2, this would form a pentenary complex of agarose–streptavidin–biotin–RNA–protein. In situations such as when the IRE is used as an affinity hook, multiple proteins may bind to the RNA (e.g., IRP1 and IRP2). For instance, we have preseparated IRP1 from IRP2 and (most other cellular components) by conventional techniques (14). The proteins are eluted by a step gradient using buffer A + 900, 1350, and 1800 mM KCl. Both IRP1 and IRP2 elute in the 1350 mM KCl fraction. In theory, IRP2 can be purified by a similar protocol, providing that it has been previously separated from IRP1 by other means.

We would recommend against using this method to purify RNA-binding proteins from crude lysates, due to the likely presence of endogenous ribonucleases in lysates.

RESULTS AND DISCUSSION

Standard EMSA techniques utilize standard TBE buffer at pH 8.3. This proved inadequate when attempting to separate biotinylated S-IRE from IRP1-S-IRE complexes because they migrate with almost equal rates (Fig. 3, left). Also, the lanes were reproducibly "blotchy" and less defined, even in free RNA lanes. It is often taught that the origin of the complex mobility shift is the added mass of the protein(s) to the RNA or DNA. While protein size is a component, the separation of free RNA from protein-complexed RNA is also a function of shape and charge (9). Thus, we reasoned that lowering the pH from 8.3 to 7.5 or 7.0 might protonate IRP1 and reduce its negative charge. Titration of the RNA in this range is not expected to be a problem since the pK_a values of adenosine ($pK_a \cong 6–7$) and cytidine ($pK_a \cong 6.3$) are lower than the buffer pH used (15). Thus, at pH 7 or 7.5, the complexes would be expected to migrate more slowly than free RNA. As shown in Fig. 3 (lanes 6–8), better separation does, indeed, occur at pH 7.5. We have also performed these EMSAs at pH 7.0 and found results similar to those shown for pH 7.5 (data not shown). Note also that in lane 8 there is no detectable free RNA, indicating that the biotinylated S-IRE is saturable. Despite this observation, it still remains to be determined if the biotin groups on the RNA affect the affinity of proteins, and will probably differ for different proteins. Because of this, we reiterate that this, as well as any EMSA, should be considered a qualitative assay unless verified by more thermodynamically rigorous methods (2).

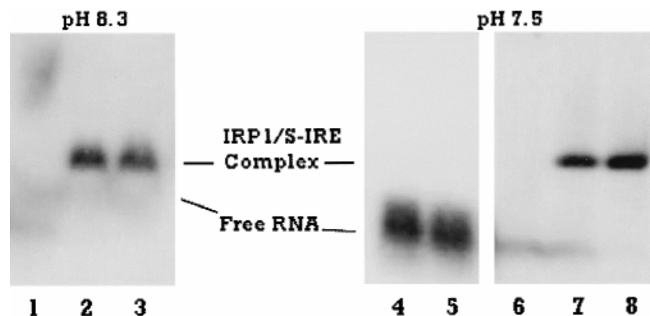

FIG. 3. Comparison of EMSAs at pH 7.5 vs pH 8.3. The data for both panels were obtained by autoradiography and film was scanned digitally. The left panel shows the results of EMSAs obtained at pH 8.3 with 1 fmol of S-IRE and 0, 2, and 4 μl of partially purified IRP1 in lanes 1, 2, and 3, respectively. The right panel shows the results of EMSAs obtained at pH 7.5. Lanes 4 and 5 contain 2 fmol of β-actin RNA + 0 and 2 μl of partially purified IRP1, respectively. Lanes 6–8 contain 1 fmol of S-IRE + 0, 2, and 4 μl of partially purified IRP1, respectively.

To verify that the interactions detected in Fig. 3 (lanes 2–3 and 7–8) are specific, we have performed EMSAs using partially purified IRP1 and β-actin RNA. Lanes 4–5 show results of this experiment. No detectable shift is seen in lane 5, which is expected, since actin contains no sequences with homology to the IRE.

We next performed titrations of S-IRE with purified IRP1 with analysis by our EMSA technique. Figure 4

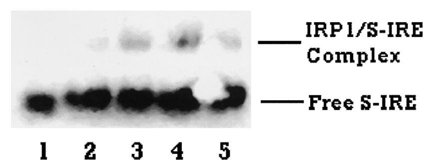

FIG. 4. Representative EMSA data for the binding of purified IRP1 to biotinylated S-IRE RNA. The data were obtained by digital photography using a cooled CCD camera and image inversion. Lanes 1–4 contain 2 fmol of S-IRE RNA + 0, 1, 2, and 4 μl of purified IRP1, respectively. Lane 5 contains 2 fmol of biotinylated S-IRE RNA + 2 μl of IRP1 + 20 fmol of nonbiotinylated S-IRE RNA as a "cold" competitor.

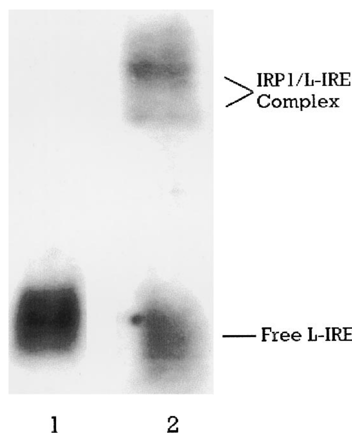

FIG. 5. Representative EMSA data for the binding of purified IRP1 to biotinylated L-IRE RNA. The data were obtained by digital photography using a cooled CCD camera and image inversion. Lanes 1 and 2 contain 2 fmol of L-IRE RNA + 0 and 2 μl of purified IRP1, respectively.

shows an example of these titrations. Lanes 1–4 contain 2 fmol of biotinylated S-IRE RNA and 0, 1, 2, and 4 μl of IRP1, respectively. These lanes show the ability of the agarose gel to precisely separate protein/RNA complexes from free RNA, as well as provide a qualitative measure of the amount of IRP1 present in each lane. Lane 5 is a competition experiment with 2 fmol of the labeled S-IRE, 20 fmol of unbiotinylated S-IRE, and 2 μl of IRP1. The addition of the unlabeled competitor decreases the protein binding with the labeled RNA, resulting in a decreased intensity in the shifted complex (compare to lane 3). This indicates that competition experiments with unlabeled RNA molecules could also be used to qualitatively determine relative protein–RNA equilibrium affinities.

Figure 5 shows that the electrophoretic mobility of long RNA fragments can also be shifted appreciably upon binding IRP1. L-IRE is shown to migrate in the agarose gel with reasonably focused resolution, both free and as a protein/RNA complex. Both lanes contain 2 fmol of biotinylated L-IRE RNA. Lane 2 also contains 2 μl of IRP1, which accounts for the shift. We believe the doublet in the IRP1/L-IRE complex band in lane 2 is due to different folds of the L-IRE that each contain a properly folded IRE. When analyzing the free bands in detail, a slight doublet can be detected during quantitative analysis (data not shown). Thus, agarose is only marginally useful for detecting conformational differences in free RNA.

This technique has also been employed with very little modification to study protein–DNA interactions using crude cell lysates, generating well-resolved complexes (N. DiIullio, personal communication). Given proper optimization of conditions (e.g., ions, pH, temperature), we believe that any protein–DNA or –RNA complex, large or small, should be theoretically amenable to qualitative study by our chemiluminescent agarose EMSA technique. As an added benefit of this technique, the biotinylated RNA or DNA can also be used to purify those protein complexes.

There are many other systems of study that could be improved through use of our chemiluminescent EMSA technique. We offer one specific example of a protein–DNA interaction that has been reported in the literature where we believe our technique would have been superior to traditional EMSAs. The binding of the transcription factor Bbf with its operator sequence has been studied by traditional EMSA (16). This interaction is important for, among other things, activation of the ferritin H-chain transcription. The Bbf protein itself can be activated for binding by a coactivator called p300, which has been postulated to form a ternary complex with Bbf and the operator DNA (16). Although detection of the Bbf–DNA interaction was tenable by traditional EMSA, the Bbf–p300–DNA ternary complex (predicted to be in excess of 400 kDa) proved too large for that technique. Thus, the existence of the ternary complex could only be inferred by use of anti-p300 immunoprecipitation followed by disruption of the Bbf–p300 complex before a second round of traditional EMSA analysis. We believe that a properly optimized chemiluminescent agarose EMSA would have been able to detect and discriminate between Bbf–DNA and Bbf–p300–DNA interactions in one gel.

Chemiluminescent agarose EMSAs are also be predicted to be useful for protein–DNA and –RNA interactions where multiple binding sites on the nucleic acid are present, such as *Escherichia coli* single-stranded binding protein (ssb), eukaryotic poly(A) binding protein, or hnRNA binding proteins. Visualization of discretely shifted complexes as a function of increasing protein concentration would make possible qualitative assessment of binding cooperativity in systems where the complexes are too large to study using traditional EMSAs.

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