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Notes & Tips

Using reverse electrophoretic mobility shift assay to measure and compare protein–DNA binding affinities

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Transcription factors regulate gene expression by binding to specific target sites in the DNA sequence of a genome. One important parameter in determining what sequences will be bound by a given transcription factor is the binding affinity of the protein for the sequence in question. Measuring the affinity is often carried out in vitro by electrophoretic mobility shift assay (EMSA)[1] [1]. In this technique, cell extracts or recombinant proteins are incubated with a labeled DNA probe, and the complexes are separated by gel electrophoresis. To evaluate the binding constant of a protein for a specific DNA sequence, a given amount of probe is incubated with increasing amounts of protein. This approach has three prerequisites: (i) that the exchange rate be sufficiently low, (ii) that the DNA binding protein be pure, and (iii) that its concentration be known.

We devised a rapid method for the measurement of protein–DNA affinity constants that bypasses both the purification and titration steps. This method takes advantage of a variant EMSA procedure, first described by Hope and Struhl [2], in which an unlabeled nucleotide probe is mixed with a radioactively labeled protein. As in the EMSA assay, the free protein and the complex will have different mobility properties in an electric field, allowing for their separation in a gel. Because the only difference with EMSA is that the protein—not the DNA—is labeled, we refer to this technique as reverse EMSA (rEMSA). Here we show that rEMSA has several advantages over classical EMSA and can be used to easily calculate protein–DNA binding constants. We use as an example ZBTB4, a human zinc finger transcription factor [3].

A first application of the method is to measure the affinity of a protein for a DNA sequence. An important feature of the technique is that knowledge of the protein concentration is not needed for the calculations. When the probe is in excess relative to the protein, one can write the following equation: $[\text{Prot:DNA}]/[\text{Prot}]_T = [\text{DNA}]/k + [\text{DNA}]$, where [Prot:DNA] is the concentration of complexes, $[\text{Prot}]_T$ is the total concentration of the given protein, [DNA] is the DNA probe concentration, and k is the affinity constant of the protein for the target site. The left-hand member is a dimensionless number that tends to 1 for saturating concentrations of DNA probe. Thus, standardizing the signal relative to the maximum value gives an expression depending only on [DNA], which is a known quantity, and on k, the parameter to be estimated. Importantly, $[\text{Prot}]_T$ is the concentration of functional proteins potentially able to bind the target DNA, even if this involves some modification of the protein (e.g., phosphorylation). It follows, in particular, that misfolded proteins are mathematically excluded from the analysis.

ZBTB4 binds methylated CpG [3], and we used rEMSA to measure the affinity of ZBTB4 for this target. We cloned the zinc finger domain of the human gene ZBTB4 in pCITE-4a (Novagen) and synthesized it in 50 μl TNT rabbit reticulocyte lysate (Promega). We used the provided amino acid mix without methionine and added 2 μl Redivue ^{35}S-methionine (Amersham) to label the translation products. We designed complementary oligonucleotides containing a methylated CpG (Proligo) and annealed them at a final concentration of 10 μM in 50 mM NaCl. Complexes were then assembled as follows. First, 12 μl of 2× assembly buffer (50 mM Hepes

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[1] *Abbreviations used*: EMSA, electrophoretic mobility shift assay; rEMSA, reverse EMSA; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; TAE, Tris acetate.

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[pH 7.5], 200 mM KCl, 2 mM ethylenediaminetetraacetic acid [EDTA], 20 mM $MgCl_2$, 0.2% NP-40, 10% glycerol, 2 mM dithiothreitol [DTT]), 1 μl of 1 mg/ml bovine serum albumin (BSA), and 8 μl double-stranded DNA at various concentrations were mixed on ice, and then 3 μl translation product was mixed in each tube. Complexes were assembled for 60 min on ice while a TAE (Tris acetate)/6% acrylamide gel was prerun at 200 V at 4 °C. The gel was loaded, run for 5 h at 200 V, dried, and scanned on a Phosphorimager (Molecular Dynamics). The type of image typically obtained in rEMSA is depicted in Fig. 1A.

Quantitation was carried out with ImageJ (http://rsb.info.nih.gov/ij), and estimation of the parameters was done by nonlinear least squares on *R* (www.r-project.org). Instead of taking the intensity of the rightmost lane as the standardizing quantity, we estimated this value from the data and then divided all of the measured intensities by this estimated maximal value. As shown in Fig. 1A, the model fitted the data reasonably and gave an estimated value of $k = 4.2\ \mathrm{nM}^{-1}$. This is a plausible figure given that the affinity of MeCP2 for methylated CpGs was estimated to be between 1.3 and 14.7 nM^{-1} [4,5].

Another use of rEMSA is to directly compare the affinities of one given protein for different sequences. ZBTB4 is a bimodal protein [3] because it binds methylated CpG and also the Kaiso binding site (KBS) TCCTGCNA [6]. To compare binding to these two sequences, we assembled the protein–DNA complexes in the presence of two oligonucleotides of different lengths: one containing the KBS and the other containing a methyl-CpG. In writing the association equations, we find the following: [ZBTB4:meCpG]/[ZBTB4:KBS] = k_{meCpG} [meCpG]/k_{KBS} [KBS], where [meCpG] is the concentration of the oligonucleotide containing methyl-CpG, [KBS] is the concentration of the oligonucleotide containing the KBS, k_{meCpG} and k_{KBS} are the affinity constants for these oligonucleotides, and [ZBTB4:meCpG] and [ZBTB4:KBS] are the respective concentrations of complexes with the oligonucleotides. We mixed the oligonucleotides at different ratios and ran the reaction as before (Fig. 1B). Fitting the model gave an estimated value of $k_{\mathrm{meCpG}}/k_{\mathrm{KBS}} = 14.1$; thus, ZBTB4 has an affinity roughly 14-fold greater for methylated CpGs than for the KBS. Note that this value was obtained independently of the first experiment; thus, this result is robust relative to the actual affinity constants of ZBTB4 for either of these targets.

rEMSA allows rapid determination and comparison of protein–DNA binding constants and can be applied to proteins produced in vitro. Although less sensitive than EMSA due to the nature of ^{35}S radioactivity, rEMSA is probably more robust given that only functional, properly folded proteins are taken into account. The field of applications, presented here for a bimodal transcription factor, also extends to the study of proteins with degenerate binding sites. We believe it will be a tool complementary to EMSA in the study of eukaryotic transcription factors.

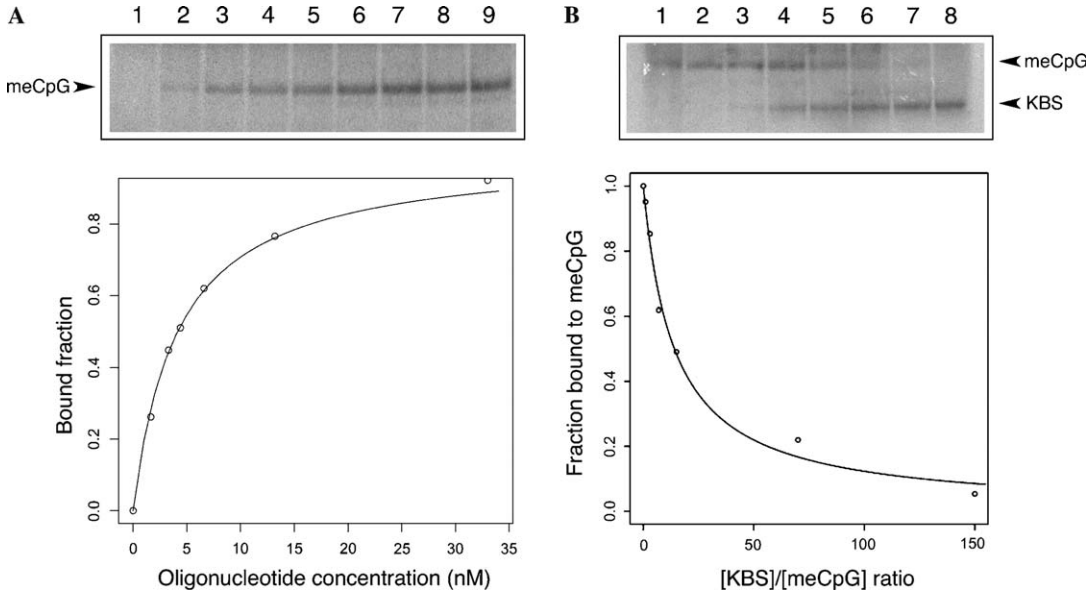

Fig. 1. (A) Determination of the affinity of ZBTB4 for methylated DNA. Top panel: Autoradiogram of the rEMSA experiment. Here, 0 nM (lane 1), 1.7 nM (lane 2), 3.3 nM (lane 3), 4.4 nM (lane 4), 6.7 nM (lane 5), 13.3 nM (lane 6), 33.3 nM (lane 7), 0.33 μM (lane 8), or 1.67 μM (lane 9) of a methylated double-stranded oligonucleotide was added to the assembly reaction, and the gel was run as explained in the text. The arrowhead on the side indicates the position of the protein–DNA complexes. Bottom panel: Quantitation. Because the peak in lane 8 was significantly lower than the peak in lane 7, this point was excluded from the analysis. Only the first seven points are shown on the graph for clarity. A value of $k = 4.2\ \mathrm{nM}^{-1}$ gave the best fit. (B) Comparison of the relative affinity of one protein for two sequences. Top panel: Autoradiogram of the rEMSA carried out with two DNA probes. The methylated oligonucleotide used previously and an oligonucleotide containing a KBS were mixed in a ratio of 1:1 (lane 2), 1:3 (lane 3), 1:7 (lane 4), 1:15 (lane 5), 1:70 (lane 6), or 1:150 (lane 7), or were not mixed (lanes 1 and 8), and were added to the assembly reaction. The arrowheads on the side indicate the nature of complexed DNA. Bottom panel: Quantitation. A value of $k_{\mathrm{meCpG}}/k_{\mathrm{KBS}} = 14.1$ gave the best fit.

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