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Note

Design of a Fluorescent Electrophoretic Mobility Shift Assay Improved for the Quantitative and Multiple Analysis of Protein-DNA Complexes

Takuo ONIZUKA,† Sumiyo ENDO, Masahiko HIRANO, Shozo KANAI, and Hideo AKIYAMA

Biological Sciences Department, Toray Research Center, Inc., 1111 Tebiro, Kamakura, Kanagawa 248-8555, Japan

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We describe a protocol for the fluorescent electrophoretic mobility shift assay improved for the quantitative analysis of protein-DNA complexes. Fluorescent-labeled oligonucleotide probes incubated with nuclear proteins were followed by electrophoresis. The signals for protein-DNA complexes were measured and normalized with fluorescent-labeled marker using fragment analysis software. This assay proved reliable measurement and multiple detection of DNA binding proteins.

Key words: electrophoresis; fluorescence; DNA-binding proteins

Transcription of genes in various types of cells is controlled by DNA-binding proteins, which bind to the regulatory regions of the genes. Measurement of these DNA-binding proteins is important in understanding the regulatory mechanism of the gene, and can provide clues to genes the function of which is unknown. The most widely applied technology for detecting sequence-specific DNA-binding proteins, such as transcription factors, has been the electrophoretic mobility shift assay (EMSA).[1–3] The assay is based on the observation that protein-DNA complexes migrate through a nondenaturing polyacrylamide gel more slowly than free DNA fragments. However the conventional EMSA involving a preparation of ^{32}P-labeled DNA probe is hazardous, and limits the number of samples that can be analyzed. Moreover nonisotopic methods reported so far do not provide reliable measurements for analysis of DNA-binding proteins.[4–7] In this work we describe a protocol for the fluorescent electrophoretic mobility shift assay improved for the measurement of protein-DNA interaction. The improved method provided reliable data and multiple detections of different DNA-binding proteins in a single lane, allowing the analysis of a desired transcription factor with a quality control simultaneously.

Octamer binding protein, oct-1, which regulates a number of genes including the housekeeping genes, was used to validate the improved EMSA. Nuclear extracts were prepared from cultured a human monocytic leukemia cell line, THP-1 cells, using NER-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, Illinois, USA) according to the procedure described by the manufacturer. Protein concentrations in nuclear extracts were measured by the Bradford assay.[8] An oligonucleotide corresponding to the consensus oct-1 binding site, 5′-TGTCGAATGCAAATCACTAGAA-3′ and its complementary oligonucleotide, was labeled with 4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein (HEX), and used for oct-1 detection and measurement. These oligonucleotides were annealed to generate double-stranded DNA fragments containing the binding sites of interest. The DNA-binding reactions were done at room temperature for 20 min. Ten ml of reaction mixture contained gel-shift binding buffer (1 mM $MgCl_2$, 0.5 mM EDTA, 4% glycerol, 0.5 mM dithiothreitol, 50 mM NaCl, 0.05 mg/ml poly(dI-dC)·poly(dI-dC), and 10 mM Tris-HCl, pH7.5), 160 fmol of the HEX-labeled oligonucleotide probe, and nuclear extracts from 1.2 μg to 6 μg. After reaction, products were mixed with a standard marker, GS2500 [Rox] (Applied Biosystems, Foster City, CA, USA), to correct sample variations. Samples were put onto a 10% nondenaturing polyacrylamide gel in 0.25xTBE buffer, and electrophoresed at 500 V with a 377 automated DNA sequencer (Applied Biosystems). During electrophoresis, temperature was kept at 23°C by circulating the cooling water from the outer pump. The signals for protein-DNA complexes were detected and peak areas were integrated using the GeneScan 2.1.1software (Applied Biosystems). The quantitative data were normalized by the value of peak area for the standard marker.

We did the experiments to evaluate the signal intensity of the sequence-specific bands in response to the protein content from the nuclear extracts for quantitative analysis. Figure 1a shows a typical gel

† To whom correspondence should be addressed. Fax: +81-467-32-0414; E-mail: Takuo_Onizuka@trc.toray.co.jp

Abbreviations: 6-FAM, 6-carboxyfluorescein; EMSA, electrophoretic mobility shift assay; HEX, 4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein

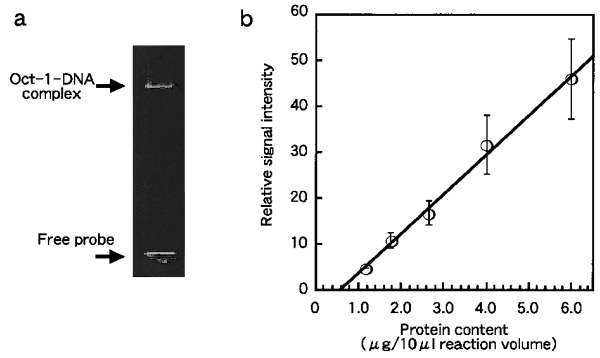

Fig. 1. Measurement of Oct-1-DNA Complex by the Fluorescent Electrophoretic Mobility Shift Assay.

Gel image of the fluorescent electrophoretic mobility shift assay (a), and linear relationship between the content of nuclear extracts and the relative signal intensity of oct-1-DNA complex (b). Nuclear extracts of THP-1 cells from 1.2 μg to 6.0 μg were mixed with a HEX-labeled oligonucleotide probe containing an oct-1 binding site and the standard marker. The reaction mixture was analyzed by fluorescent electrophoretic mobility shift assay as detailed in the text. Each plot was normalized by the value of the standard marker. Data represent mean $\pm$ S.D. obtained from three independent measurements.

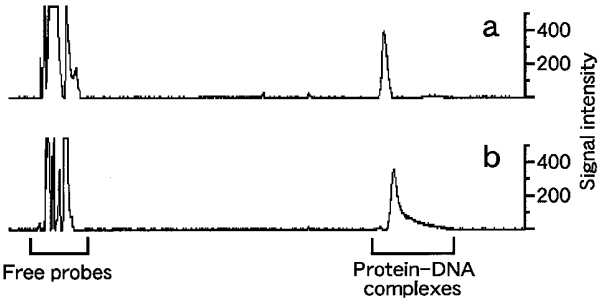

Fig. 2. Fluorescence Profiles for Simultaneous Analysis of Two Transcription Factors, NF-κB (a) and Oct-1 (b), in a Single Lane.

Two kinds of fluorescent colors, 6-FAM and HEX, were used for labeling oligonucleotide probes containing the NF-κB or oct-1 binding site, respectively. The probes were mixed with 3 μg of nuclear extracts from THP-1 cells stimulated with lipopolysaccharide (1 μg/ml) for 1 h and put on a 10% non-denaturing polyacrylamide gel.

image of a specific oct-1-DNA complex by the fluorescent electrophoretic mobility shift assay. The specificity of the DNA-binding protein is confirmed by competition experiments in which the formation of protein-DNA complex was specifically competed by a 100-fold molar excess of non-labeled specific probe, but not by a non-specific oligonucleotide probe (data not shown). A nearly linear relationship between the amount of input nuclear extracts and the relative signal intensity of the sequence-specific band for oct-1 was observed (Fig. 1(b)). Protein-DNA complexes within the gel are very stable because of the "caging effect" due to the gel matrix, which may prevent dissociated components from diffusing away and promote recombination with each other.[9] Therefore the equilibrium between protein-DNA complexes and the dissociated components may be established within the gel. We did the experiment with the DNA probe in large excess over the DNA-binding protein, resulting in a proportional correlation between the signal intensity for the protein-DNA complexes and the concentration of the DNA-binding protein, as shown in Fig. 1(b). Thus, compared to the other non-isotopic EMSA methods,[4-7] our improved assay can measure the desired protein-DNA complexes accurately by calculating peak areas of fluorescence intensity corresponding to gel-retarded protein-DNA complexes including a standard marker.

When we study DNA-binding proteins that bind to a proximal region of a given gene, it is preferred to detect the standard transcription factor as an internal control simultaneously. In the conventional EMSA, it is difficult to distinguish a particular DNA-binding protein from several ones in a single lane, especially when the molecular sizes are similar. However, by using the fluorescent-labeled probe instead of a radioactively labeled one, we can detect a specific band among several other ones by the application of different fluorescent colors. In this experiment, two kinds of fluorescent colors, 6-carboxyfluorescein (6-FAM) and HEX, were used for labeling nuclear factor kappa B (NF-κB) and the oct-1 oligonucleotide probes, respectively. The following sequence was used as an NF-κB specific probe: 5′-GCTCATGG-GTTTCTCCAC-3′ (Only a single-stranded oligonucleotide is shown). The probes were mixed with 3 μg of nuclear extracts, and electrophoresed. Figure 2 shows the simultaneous analysis of transcription factors NF-κB and oct-1 in a single lane. Oct-1 is thought to be the endogenous ubiquitous transcription factor.[10] Hence the simultaneous analysis of transcription factors with oct-1 can immediately identify false-negative samples, and oct-1 can serve as a quality control, demonstrating more reliable results.

This study has introduced an improved EMSA using fluorescent labeling and a DNA sequencer. This assay provides the following advantages: I) simplicity in procedure, allowing the analysis of a large number of samples simultaneously, II) use of a fluorescent-labeled probe which is highly stable and can be used repeatedly, III) the ability to use multiple fluorescent-colored probes simultaneously, allowing the analysis of multiple factors with a quality control in a single experiment, IV) speed and sensitivity in providing measurements regarding the protein-DNA complexes of interest. These advantages are highly favorable when we investigate the activation or inhibition of transcription factors under various conditions.

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