Research Report

Multiple Transcription Factor Profiling by Enzyme-Linked Immunoassay

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**Z. Shen, J. Peedikayil,
G.K. Olson, P.D. Siebert,
and Y. Fang**
BD Biosciences Clontech,
Palo Alto, CA, USA

ABSTRACT

Transcription factor-DNA interactions have been widely studied in the regulation of gene expression. We have established an enzyme-linked immunoassay platform to quantify specific transcription factor-DNA interactions. In our assay, dsDNA immobilized on a 96-well plate captures the transcription factor from the nuclear extract of mammalian cells. The DNA-bound transcription factor is detected and quantified by enzyme-linked immunoassay using a transcription factor-specific antibody. We have profiled multiple transcription factors involved in inflammation including NF κ B p50, NF κ B p65, c-Rel, c-Fos, CREB-1, and ATF-2. When compared with the traditional electrophoretic mobility shift assay, the enzyme-linked immunoassay shows a 10-fold higher sensitivity, eliminates the use of radioactivity, allows for a high-throughput format, and is faster.

INTRODUCTION

Transcription factors, a subset of DNA binding proteins, are at the heart of the control of gene expression (12). Changes in gene expression patterns are the result of variations in the abilities of transcription factors to bind to DNA. Therefore, investigations into DNA transcription factor interactions constitute one of the most rapidly growing areas of molecular biology and can offer powerful insights for drug target discovery and validation (2,5).

There are several approaches available to study DNA-protein interactions (4,6,15). One approach explores the ability of a transcription factor to bind to a known *cis*-acting DNA element upon stimulation. For this approach, *in vivo* reporter assays and the *in vitro* electrophoretic mobility shift assay (EMSA or gel-shift assay) are the most commonly used methods (3,13).

Traditionally, the EMSA has been one of the most powerful tools for studying the ability of a transcription factor to bind DNA. However, the EMSA has several limitations, including the need for radioactive materials, a limit to the number of samples that can be run, and a long assay time. These limitations and the desire for a high-throughput format led to the development of an enzyme-linked DNA-protein binding assay (1,9). Both Hibma et

al. (9) and Benotmane et al. (1) developed an assay based on ELISA to detect the binding of purified proteins to specific DNA immobilized on a 96-well microplate.

In this report, we have established a generic assay platform for detecting DNA-protein binding activity using purified protein and nuclear extracts from mammalian cells. Moreover, we have expanded the assay to profile multiple transcription factor-DNA interactions that play an important role in many physiological responses and pathological events, including inflammation and oncogenesis.

MATERIALS AND METHODS

Oligonucleotides

The parent and complementary single-stranded oligonucleotides, corresponding to the wild-type and mutated transcription factor consensus sequences (Table 1), were purchased from Operon Technologies (Alameda, CA, USA). Each oligonucleotide was purified using HPLC, and the parent strand was biotinylated at the 5' end by Operon Technologies. Before use, both strands were annealed by heating at 100°C in TE buffer (10 mM Tris-Cl, pH 7.6, 1 mM EDTA) for 10 min and gradually cooled to room temperature.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Nuclear Extract and Purified Protein

Nuclear extracts were made using the TransFactor Extraction Kit (BD Biosciences Clontech, Palo Alto, CA, USA). Cells were grown to 80%–90% confluency and were either non-induced, HeLa and Jurkat, or induced, HeLa. For induction, HeLa cells were incubated with either 0.1 $\mu\text{g/mL}$ TNF- α (BD Biosciences Clontech) for 30 min or 2 $\mu\text{g/mL}$ PMA (Sigma, St. Louis, MO, USA) for 2 h. Cells were harvested, washed, and pelleted at $450\times g$. The cells were resuspended in 5 volumes of hypotonic lysis buffer, dependent on the pelleted cell volume, and incubated on ice for 15 min. The cells were then centrifuged at $420\times g$, resuspended in 2 volumes of hypotonic lysis buffer based on the original pelleted cell volume, and disrupted with a syringe. The disrupted cells were centrifuged at $11\,000\times g$ to isolate the cytoplasmic fraction, which was removed and stored at -70°C . The pellet containing the nuclei was resuspended in extraction buffer, disrupted with a syringe, and gently shaken for 30 min at 4°C . Finally, the material was centrifuged for 5 min at $21\,000\times g$, and the supernatant corresponding to the nuclear extract was removed and stored at -70°C . Purified human recombinant NF κ B p50 was purchased from Promega (Madison, WI, USA).

Transcription Factor Enzyme-Linked Immunoassay (TF-EIA)

NeutrAvidin^{TM}-coated 96-well strip plates (Pierce Chemical, Rockford, IL, USA) were incubated with 100 μL /well of 33 nM biotinylated dsDNA, corresponding to related wild-type and mutated consensus sequences, in TransFactor buffer (BD Biosciences Clontech) for 1 h at room temperature. After each step, three washes were performed. Each well was then blocked with 3% nonfat milk in TransFactor buffer for 1 h. Either nuclear extract or purified transcription factor diluted in TransFactor buffer plus 3% nonfat milk was added at a volume of 50 μL /well and incubated for 1 h at room temperature. Primary antibody diluted in TransFactor buffer plus 3% milk (Table 1) was then added at 100

μL /well and incubated at room temperature for 1 h. After washing, 100 μL /well of secondary antibody diluted in TransFactor buffer with 3% milk (1:1000) was added and incubated at room temperature for 30 min. Finally, after the addition of 100 μL /well of TMB substrate, color development was detected at 655 nm with a Bio-Rad® model 550 microplate reader (both from Bio-Rad Laboratories, Hercules, CA, USA).

Antibodies

The primary antibodies used include anti-NF κ B p50 (Upstate Biotech, Waltham, MA, USA), anti-NF κ B p65 (Upstate Biotech), anti-c-Rel (Santa Cruz Biotech, Santa Cruz, CA, USA), anti-c-Fos (Santa Cruz Biotech), anti-CREB-1 (Santa Cruz Biotech), and anti-ATF-2 (Santa Cruz Biotech). The secondary antibody used was anti-rabbit IgG-HRP (BD-Transduction Labs, Lexington, KY, USA).

Oligonucleotide Competition Assay

Wells were incubated with wild-type dsDNA oligonucleotides in TransFactor buffer for 1 h at room temperature. Increasing amounts (25–200 ng) of wild-type or mutant competition oligonucleotides were added to 30 μg nuclear extract in 50 μL total volume of TransFactor buffer plus 3% milk. After blocking, this mixture was then added to each well coated with the wild-type dsDNA for 1 h. The remaining steps of the TF-EIA were then performed as previously described.

EMSA

The dsDNA oligonucleotide (wild-type) was labeled with ^{32}P using a 3'-end labeling kit (Amersham Biosciences, Piscataway, NJ, USA). Either nuclear extract or purified transcription factor was incubated with 2.5 μL ^{32}P -oligonucleotide probe for 20 min in 20 mM HEPES (pH 7.9), 40 mM KCl, 1 mM MgCl_2 , 100 μM EDTA, 500 μM dithiothreitol, 6% glycerol, and 0.1 mg/mL poly dI-dC. The samples were then fractionated on a $0.5\times$ TBE, 5% acrylamide gel. For supershift analysis, 0.5 μL polyclonal antibody or 1 μg

monoclonal antibody were incubated with ^{32}P -labeled DNA-transcription factor complex.

RESULTS AND DISCUSSION

Principle of the TF-EIA

Wild-type and mutated dsDNA consensus sequences for each transcription factor were immobilized on neutravidin-coated 96-well plates. The DNA-coated wells were then incubated with purified protein, mammalian nuclear extract, or mammalian cellular extract. DNA-transcription factor complexes were detected with primary antibodies specific for the target transcription factors and an HRP-conjugated secondary antibody. Finally, the TMB substrate was added to the wells, and color development was measured with a microplate reader (Figure 1).

Increased Sensitivity of the TF-EIA Compared with the EMSA

The sensitivities of the TF-EIA and EMSA to detect DNA-protein binding activity were compared using purified recombinant human NF κ B p50 protein. Neutravidin-coated wells were incubated with wild-type NF κ B p50 dsDNA (Table 1), at the saturating concentration of 33 nM. The dsDNA was then exposed to purified NF κ B p50 protein at concentrations in the range of 0–25.6 nM. Anti-NF κ B p50 antibodies detected NF κ B p50 protein that bound to the dsDNA. The result was a sigmoidal curve with the saturation plateau at 6 nM (Figure 2a). We defined the lowest detection point to be the concentration corresponding to two times the background absorbance, which was 0.3 nM purified NF κ B p50 protein.

^{32}P -end-labeled NF κ B p50 wild-type dsDNA was incubated with increasing amounts of purified NF κ B p50 protein from 0–102.4 nM. The free and protein-bound dsDNA was separated by gel electrophoresis, and the absorbance of the bands was determined on the Storm^{TM} 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA) (Figure 2b). The lowest detected concentration based on two times the background, replacing absorbance with

absorbance of the band, was approximately 3 nM. From this point, the band intensity gradually increased until it reached a saturation point at approximately 100 nM. At high concentrations of purified protein, increasing amounts of multimeric forms were seen. These bands were included with the lower band in the determination of absorbance to compare with the TF-EIA, which has no way to differentiate protein-DNA binding forms such as monomers or oligomers.

As seen in Figure 2a, after normalization, both the TF-EIA and the EMSA showed similar sigmoidal curves. However, based on the lowest detected protein concentration of both assays, the TF-EIA (0.3 nM) exhibited a 10-fold higher sensitivity than the EMSA (3 nM). One explanation for this increased sensitivity is the differences in how the local environments of the two assays act

Figure 1. The principle of the TF-EIA. dsDNA immobilized on a 96-well plate captures the transcription factor from the nuclear extract. A transcription factor-specific antibody detects the DNA-bound transcription factor. This complex is then quantified by the combination of an HRP-conjugated antibody and its substrate.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Table 1. Transcription Factors, Consensus Sequences, and Primary Antibody Dilutions Used in This Study

Transcription Factors	DNA Consensus Sequence	Primary Antibody Dilution	Reference
NFκB p50, wt	GGGGATCCC	1:5000	10
NFκB p50, mut	GCQGATCCC		
NFκB p65/c-Rel, wt	GGGGTATTTC	1:500/1:100	14
NFκB p65/c-Rel, mut	GGCATAGTCC		
c-Fos, wt	TGACTCA	1:100	7
c-Fos, mut	TGACTTG		
CREB-1/ATF-2, wt	TGACATCA	1:100/1:100	11
CREB-1/ATF-2, mut	GAACATAG		

on the transcription factor-DNA interaction. We hypothesize that a subset of the DNA-protein complexes may be interrupted while moving through the gel

matrix, whereas the same subset remains in a soluble, more physiological form in the microplates. This was also observed previously by Benotmane et al. (1).

Figure 2. Sensitivity comparison of the TF-EIA and EMSA. (a) TF-EIA: Purified NFκB p50 protein in increasing concentrations (by a factor of two) from 0–25.6 nM was incubated with NFκB p50 wild-type dsDNA. EMSA: Purified NFκB p50 protein in increasing concentrations (by a factor of two) from 0–102.4 nM was incubated with ³²P-end-labeled NFκB p50 wild-type dsDNA. (b) Gel visualization of the EMSA sigmoidal curve. Lane 13 is a supershift using anti-NFκB p50 polyclonal antibody incubated with ³²P-end-labeled NFκB p50 wild-type dsDNA and 12.8 nM NFκB p50-purified protein, which corresponds to the concentration used in lane 9.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Analysis of Transcription Factor-DNA Binding Activity in Mammalian Nuclear Extract

In many cases, purified protein is either unavailable or difficult to purify, so we tested the ability of the TF-EIA to detect specific transcription factor-DNA binding using nuclear extract of mammalian cells. NF κ B p50 wild-type and mutant dsDNA consensus sequences (Table 1) were incubated with increasing amounts of nuclear extract (0–30 μ g) from HeLa cells induced with TNF- α (Figure 3a). The protein binding to wild-type dsDNA increased proportionally with the amount of nuclear extract applied. Meanwhile, no increase in binding was observed in the wells coated with mutant dsDNA.

Typically, an oligonucleotide competition assay is performed in the EMSA to assess binding specificity and to determine the key bases in the protein-binding DNA consensus sequence. The same competition assay was performed in the TF-EIA. Non-biotinylated oligonucleotides corresponding to the wild-type or mutant NF κ B p50 consensus sequences were mixed with 30 μ g HeLa induced with TNF- α nuclear extract (the highest dose amount), and this was then added to wells coated with the biotinylated wild-type NF κ B p50 dsDNA (Figure 3a). With the addition of increasing amounts of wild-type competitor oligonucleotide, we observed a gradual decrease of DNA-protein binding activity, while no corresponding decrease was seen with the addition of increasing amounts of mutant oligonucleotides.

We performed the dose and competition assay with members of two other transcription factor families, ATF-2 (Figure 3b) and c-Fos (Figure 3c), using Jurkat nuclear extract and HeLa induced with PMA nuclear extract, respectively. The three transcription factors we tested all resulted in similar dose responses, and DNA-protein binding was successfully competed away with the addition of a specific wild-type oligonucleotide. In each case, DNA-protein binding was detected at the lowest dose of 5 μ g nuclear extract, and the detectable binding was almost completely abolished with the addition of 40 times competitor oligonucleotide.

Figure 3. Dose response and competition assays for NF κ B p50, ATF-2, and c-Fos. Dose response assays were performed by applying increasing amounts of nuclear extract (0–30 μ g) to either wild-type or mutant dsDNA-coated wells specific for each transcription factor. Meanwhile, in the competition assay, wild-type or mutant oligonucleotides specific for each transcription factor in increasing concentration (25–200 ng) were incubated with 30 μ g nuclear extract, and this mixture was added to wild-type dsDNA-coated wells. (a) NF κ B p50 was detected by anti-NF κ B p50 in HeLa stimulated with TNF- α nuclear extract. (b) ATF-2 was detected by anti-ATF-2 in Jurkat nuclear extract. (c) c-Fos was detected by anti-c-Fos in HeLa stimulated with PMA nuclear extract. The data represent the means of three values $\pm$ SD.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

To quantify the amount of transcription factor present in the nuclear extract, purified protein could be used to produce a standard curve. Slightly different detection sensitivities were observed for each transcription factor. These differences may be caused by the following: each nuclear extract may contain different concentrations of each transcription factor; alternatively, the binding affinities of the transcription factor for the consensus sequence and the binding affinities of the antibodies for proteins could be different.

Specificity of Antibody-Transcription Factor Binding

To verify that each antibody is bound to its specific transcription factor, the TF-EIA was performed using specific and nonspecific antibodies. NFκB p50 wild-type dsDNA-coated wells were incubated with 30 μg HeLa induced with TNF-α nuclear extract and one of three antibodies: anti-NFκB p50, anti-ATF-2, and anti-c-Fos (Figure 4). The NFκB p50 protein-DNA complex was detected by anti-NFκB p50,

but not by anti-ATF-2 or anti-c-Fos. Similarly, only the specific antibody detected ATF-2 protein-DNA complexes and c-Fos protein-DNA complexes when using Jurkat nuclear extract and HeLa induced with PMA nuclear extract, respectively. No antibody cross-reactivity was observed in this assay, which indicates that positive signals usually correspond to the specific dsDNA-protein-antibody complexes. However, protein-protein interactions among transcription factors naturally occur, such as the NFκB p50 and NFκB p65 heterodimer. In this assay, we were able to detect both NFκB p65 and NFκB p50 separately, using their respective antibodies on a plate coated only with NFκB p50 wild-type dsDNA (data not shown).

Profiling the DNA Binding Activity of Multiple Transcription Factors in Different Nuclear Extracts

It is often of interest to compare transcription factor activation in induced versus non-induced cells. The activation levels of six transcription factors involved in immunodisease were profiled simultaneously in three different HeLa nuclear extracts. Nuclear extract from HeLa, HeLa induced with TNF-α, and HeLa induced with PMA were added to wells containing wild-type or mutant dsDNA corresponding to NFκB p50, NFκB p65, c-Rel, c-Fos, CREB-1, and ATF-2 (Table 1). In all cases, the mutant dsDNA-coated wells resulted in little or no signal (data not shown).

The transcription factor NFκB is normally sequestered in the cytosol because of its association with IκB. Upon stimulation, this association is dissolved and NFκB translocates to the nucleus (16). Increased levels of both NFκB p50 and NFκB p65 were detected in induced HeLa nuclear extract when compared to non-induced HeLa nuclear extract (Figure 5).

Activation of the protein kinase C signaling pathway by PMA causes c-Fos to translocate to the nucleus (17). A significant increase in c-Fos binding activity in HeLa induced with PMA nuclear extract was observed when compared with non-induced HeLa nuclear extract (Figure 5).

Figure 4. Antibody specificity of the TF-EIA for NFκB p50, ATF-2, and c-Fos. HeLa (30 μg) stimulated with TNF-α nuclear extract was added to NFκB p50 wild-type dsDNA-coated wells and incubated with anti-NFκB p50, anti-ATF-2, and anti-c-Fos. Jurkat nuclear extract (30 μg) was added to ATF-2 wild-type dsDNA-coated wells and incubated with the same three antibodies. HeLa (30 μg) stimulated with PMA nuclear extract was added to c-Fos dsDNA-coated wells and incubated with the same three antibodies. The data represent the means of three values ± SD.

Figure 5. Multiple transcription factor profiling in different HeLa nuclear extracts. Three separate HeLa nuclear extracts (non-induced, induced with TNF-α, and induced with PMA) were applied to wells coated with wild-type dsDNA corresponding to NFκB p50, NFκB p65, c-Rel, c-Fos, CREB-1, and ATF-2. This was followed by detection with the corresponding primary antibody. The data represent the means of two values ± SD.

In this profiling experiment, we looked at a specific type of nuclear extract stimulated with different conditions to focus on transcription factors involved in inflammation. Although c-Rel and CREB-1 were not activated in these nuclear extracts (Figure 5), we saw high levels of endogenous c-Rel in non-induced Raji nuclear extract and high levels of CREB-1 in non-induced PC-12 nuclear extract (data not shown). The ATF-2 protein is ubiquitous and continually expressed (8); thus, ATF-2 exhibits high endogenous levels in all the HeLa nuclear extracts (Figure 5), along with non-induced Raji, PC-12, and Jurkat nuclear extracts (data not shown).

We have developed an alternative to the EMSA based on the ELISA platform. Compared to the EMSA, the TF-EIA has 10-fold higher sensitivity, takes a short time to run, and uses no radioactivity. Because of its sensitivity, the TF-EIA can be used to study DNA-protein binding events in nuclear extract, whole cell extract, and may be effective with tissue extract. Also, quantitative studies can be performed by using purified protein as a standard. Like the EMSA, the TF-EIA can be used for competition studies or to detect novel transcription factors. In the case of the TF-EIA, when no antibody is available, the putative transcription factor can be fused with a tagged expression vector and detected with a tag-specific antibody (data not shown).

The TF-EIA contains an inherent flexibility to screen for activation or inhibition of DNA binding protein activity in a high-throughput format and is easily expandable to 384 wells. This could be especially useful in drug discovery and cancer research. In addition, with the completion of the human genome project, more and more transcription factors will be identified; thus, an even higher capacity platform will be required. The TF-EIA can be adapted to the array format by immobilization of the dsDNA on glass and the use of an antibody cocktail. The miniaturization resulting from the microarray will require less sample and antibody to be used and accelerate the ability to analyze more transcription factor-DNA binding events simultaneously.

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Address correspondence to:

Dr. Yu Fang
BD Biosciences Clontech
1020 East Meadow Circle
Palo Alto, CA 94303, USA
e-mail: francine_fang@bd.com

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