Use of an ALFexpress™ DNA Sequencer to Analyze Protein-Nucleic Acid Interactions by Band Shift Assay

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ABSTRACT

Gel retardation analysis, or band shift assay, is technically the simplest method to investigate protein-nucleic acid interactions. In this report, we describe a nonradioactive band shift assay using a fluorescent DNA target and an ALFexpress™ automatic DNA sequencer in place of the current method that utilizes radioactively end-labeled DNA target and a standard electrophoresis unit. In our study, the dsDNA targets were obtained by annealing two synthetic oligonucleotides or by PCR. In both cases, a molecule of indodicarbocyanine (CY5) was attached at the 5' OH end of one of the two synthetic oligonucleotides, with a ratio of one molecule of fluorescent dye per molecule of dsDNA. To demonstrate the feasibility of this new band shift assay method, the DNA-binding proteins selected as models were the BlaI and AmpR repressors, which are involved in the induction of the Bacillus licheniformis 749/I and Citrobacter freundii β -lactamases, respectively.

The results show that the use of an automatic DNA sequencer allows easy gel retardation analysis and provides a fast, sensitive, and quantitative method. The ALFexpress DNA sequencer has the same limit of detection as a laser fluorescence scanner and can be used instead of a FluorImager™ or a Molecular Imager®.

INTRODUCTION

Proteins that interact with nucleic acids play a fundamental role in cell life. To highlight a DNA-binding protein and locate a protein-binding site on the DNA, electrophoretic mobility shift assays (e.g., band shift assay or gel retardation analysis) (7) and DNA footprinting analysis (3) are used. Usually, both techniques require end-labeling of the DNA, which is traditionally achieved by radioisotopes such as ^{32}P or ^{35}S . To eliminate the difficulties inherent in the detection (i.e., autoradiography and densitometry) and handling of radioactive compounds (i.e., constraining waste, the short life of the radioisotope), a stable, fluorescently end-labeled DNA can be used and the bands detected by a laser fluorescence scanner such as a FluorImager™ (Amersham Pharmacia Biotech, Roosendaal, Netherlands) or a Molecular Imager® (BioRad Laboratories, Nazareth, Belgium).

In this paper, we discuss our method involving a stable, fluorescently end-labeled DNA and an automatic DNA sequencer as an alternative to using a laser fluorescence scanner. To explore the performance of this new strategy in detecting and characterizing a DNA-binding activity, the BlaI repressor and the AmpR repressor-activator, involved in the induction of the *Bacillus licheniformis* 749/I β -lactamase (BlaP) and the *Citrobacter freundii* β -lactamase (AmpC), respectively, were selected as test proteins (1,2,5,9). The BlaI DNA-binding protein (128 amino acids) belongs to the LacI repressor family and

recognizes two similar operators, Op1_BI and Op2_BI, in the blaP promoter region (9). The AmpR DNA-binding protein (290 amino acids) belongs to the LysR family and recognizes one operator region, Op_Cf, in the inter-cistronic region between ampC and ampR (1).

MATERIALS AND METHODS

Oligonucleotides and DNA Annealing

The synthetic oligonucleotides (Amersham Pharmacia Biotech) described in this study are summarized in Table 1. To generate the double-stranded fluorescent target Op1_BI, equimolar amounts of the two complementary single-stranded oligonucleotides, Op1_BI_upper and Op1_BI_lower (Table 1), were mixed in the annealing buffer (10 mM Tris-HCl, pH 7.5, 1 mM Na₂EDTA, and 0.5 M NaCl) at final concentrations of 0.05 mM and heated for 5 min in boiling water. The Op_Cf fluorescent target was obtained in a similar way by mixing equimolar amounts of Op_Cf_upper and Op_Cf_lower (Table 1).

PCR Amplification of the *B. licheniformis* blaP Promoter Region

The *B. licheniformis* blaP promoter region containing the two DNA sequences Op1_BI and Op2_BI was amplified from the pRTW8 plasmid (6) by using *Tth* DNA polymerase (Eurogentec, Seraing, Belgium) and the

Table 1. Oligonucleotides Used in this Study

Oligonucleotide	Sequence	Length
Op1_BI_upper	5'-GAAAGTATTACATATGTAAGATTTAAATGC-3'	30 bp
Op1_BI_lower	CY5 ^a -GCATTTAAATCTTACATATGTAATACTTTC-3'	30 bp
BlaP_upper	5'-ATCCAGATCTTCCCTCCGTTATTGTCCCCG-3'	30 bp
BlaP_lower	Cy5 ^a -ATATGGATCCATCAAAATCGTCTCCCTCCG-3'	30 bp
Op_Cf_upper	Cy5 ^a GCATTCCCTGTTAGAAAAACCTTATATCTGCTGCTAAATTTAACCG-3'	44 bp
Op_CF_lower	5'-CGGTTAAATTTAGCAGCAGATATAAGTTTTTCTAACAGGAATGC-3'	44 bp
R P	Cy5 ^a -GGAAACAGCTATGACCATG-3'	19 bp

^aCY5 is the fluorescent dye modifying the 5' end of the oligonucleotide.
The inverted repeats recognized by BlaI or AmpR and present in Op1_BI and Op_CF are indicated by arrows.

amplimers BlaP_upper and BlaP_lower (Table 1). Approximately 10 ng pRTW8 were used for PCR amplification in a total volume of 500 μ L *Tth* buffer containing 200 μ M each dNTP (Amersham Pharmacia Biotech), 1 U *Tth* DNA polymerase, and 100 pmol each primer. The thermal cycler (Uno-Thermobloc; Biometra, Westburg, Leusden, Netherlands) was programmed as follows: denaturing at 95°C, annealing at 70°C for 60 s, and extension at 72°C for 60 s. This cycle was repeated 25 times. The PCR product, Op1 + Op2_BI target (260 bp), was purified using a GFXTM PCR purification kit (Amersham Pharmacia Biotech).

Source of BlaI Repressors, blaP Induction, and Cytoplasmic Extracts

BlaI wild-type (BlaIWT) and the thermosensitive BlaI (BlaITS) repressors (4) were expressed in *E. coli* BL21 DE3 (Novagen, Bierges, Belgium) by using a pET22b vector (P. Filée, unpublished data) and purified as described by Wittman et al. (8).

B. licheniformis 749/I (2) was grown in 250 mL LB broth. To induce BlaP expression, cephalosporin C (Sigma, Bornem, Belgium) was added to a final concentration of 5 μ g/mL when A_{600} of the culture reached a value of 0.8. After 2 h of induction, the cells were harvested by centrifugation at 4°C for 10 min at 5000 $\times$ g. Cells were resuspended in 1 mL 20 mM phosphate buffer, pH 7.0, 1 mM Pefabloc[®] (Roche Molecular Biochemicals, Brussels, Belgium), and disrupted by sonication (3 cycles of 30 s at

0°C with a peak-to-peak distance of 6 μ m). The supernatant containing the cytoplasmic fraction was harvested by the centrifugation of the sonicated cells at 13 000 $\times$ g for 10 min at 4°C. For non-induced cytoplasmic extracts, the same protocol was followed without the addition of the BlaP inducer.

Source of AmpR Repressors and Cytoplasmic Extracts

In this preparation, the AmpR (donated by Dr. Christine Jacobs) was purified from solubilized inclusion bodies (5). The cellular extract of *E. coli* TG1 (Stratagene, Amsterdam, Netherlands) harboring pNU305 plasmid (5) carrying *ampC* and *ampR* genes was prepared as described for *B. licheniformis* cytoplasmic extracts and was the source of native AmpR.

Quantification of Protein Concentrations

Protein concentration was estimated by reaction with 2-bicinchoninic acid (BCA) protein assay (Pierce Chemical, Antwerpen, Belgium).

DNA-Binding Assay

Synthetic fluorescent oligonucleotides and nonspecific salmon sperm DNA (Sigma) were diluted in the DNA-binding buffer: 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM Na₂EDTA, 5% glycerol, and 50 μ g DNase and BSA/mL (protease-free) (Affiland, Sart-Tilman, Belgium). Aliquots corre-

sponding to 125 or 160 μ g cytoplasmic proteins were concentrated by freeze-drying and by dissolving them in the DNA-binding buffer, supplemented with the other constituents of the DNA-binding reaction. When quantitative comparison between two samples was required, the fluorescent reverse primer (RP) (Table 1) was added as an internal reference to correct the variable electronic amplification of the different photodetectors of the ALFexpressTM DNA sequencer (Amersham Pharmacia Biotech). After 30 min of incubation at 37°C, the reaction mixture was loaded onto a 4.5% or 5.5% non-denaturing polyacrylamide gel, acrylamide/bis-acrylamide, in a 29:1 ratio (Bio-Rad Laboratories). The gel was poured into the small cassette used for restriction fragment length polymorphism (RFLP) analysis (30 x 15 x 0.05 cm) and contained the following electrophoresis buffer: 89 mM Tris base, 89 mM boric acid, and 2 mM Na₂EDTA (TBE buffer). The gel was run at constant power (10 W; endpoints for voltage and current were 800 V and 45 mA, respectively) for 120–200 min. During the electrophoresis, the temperature of the gel was kept at a constant value. The collected data were analyzed by the Fragment Manager software (Amersham Pharmacia Biotech).

RESULTS

The sensitivity of this method and its capacity to estimate an unknown quanti-

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ty of fluorescently labeled DNA were evaluated as follows. Increasing amounts of the CY-5 DNA probe, Op1_B1 (Table1), were loaded onto a 4.5% acrylamide gel and were submitted to electrophoresis on the ALFexpress DNA sequencer. Surprisingly, for an identical amount of Op1_B1, the areas of the peaks varied considerably from one detector to another. To account for the variation in the electronic amplification among the 40 photodetectors of the ALFexpress DNA sequencer, an identical quantity of CY5-RP was added to each sample as an internal standard. The peaks obtained in the different electropherograms were integrated using the Fragment Manager software, and the ratios of the areas corresponding to the labeled Op1_B1 target to those of the internal standards were computed and expressed as relative intensities. Figure 1 shows the relationship between the

relative intensity and the quantity of fluorescent probe Op1_B1. Under these conditions, 5 fmol oligonucleotides can be routinely detected.

Using the ALFexpress DNA sequencer, the *BlaI* efficiency in binding to its DNA targets, Op1_B1 and Op2_B1, was used to reproduce classic band shift assays to demonstrate the specificity of a DNA-binding protein (Figure 2) and to highlight the presence of several operators on the same DNA fragment (Figure 3). To detect the presence of a DNA-binding protein in a cellular extract, the *BlaI* and *AmpR* repressors were used, respectively (Figure 4).

To take advantage of the quantitative data collected by the ALFexpress DNA sequencer, a standard plot of the percentage of bound DNA (*BlaI*-DNA complex) to the total DNA versus the total quantity of *BlaI* was constructed (Figure 5). Under these conditions, in

Figure 1. Quantification of the fluorescent Op1_B1 target with the help of the ALFexpress DNA sequencer. Various quantities of the fluorescent target Op1_B1 ranging from 5 to 100 fmol were loaded onto a 4.5% non-denaturing polyacrylamide gel. The graph shows the ratio of the areas under the peaks to the corresponding labeled Op1_B1 target and the fluorescent RP oligonucleotide (relative intensity) versus the quantity of the Op1_B1 target.

Figure 2. Specificity of *BlaI*WT repressor for Op1_B1 target. (A) Saturation assay; the Op1_B1 target (0.5×10^{-8} M) (lane 1) was subjected to binding reactions using increasing concentrations of *BlaI*WT (3.3×10^{-9} M, 8.2×10^{-9} M, 1.6×10^{-8} M, 2.5×10^{-8} M, 3.3×10^{-8} M, 3.3×10^{-7} M, 3.3×10^{-6} M, and 3.3×10^{-5} M; lanes 2–9, respectively). In each assay, a 400-fold excess of nonspecific DNA (nsDNA) was added in the binding buffer. (B) Competition assay, with nsDNA. In this experiment, 0.1 and 10 µg salmon sperm DNA were added in the binding buffer to show the effect of nsDNA on the *BlaI*WT-Op1_B1 complex. (C) Supershift assay, before incubation with pre-immune or anti-*BlaI*WT serums. *BlaI*WT (3.3×10^{-8} M) was allowed to bind to Op1_B1 target (0.5×10^{-8} M) at 30°C for 30 min. After the addition of the serum (final dilution 1:100), the binding reaction was allowed to continue for 30 min at 30°C. Free, free DNA target; Cp, DNA-protein complex; Cp*, DNA-*BlaI*-antibody complex. The fluorescence is expressed in arbitrary units.

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the cytoplasm of non-induced *B. licheniformis* cells, 5 ng BlaI were found in 125 μ g cytoplasmic proteins or $4 \times 10^{-3}\%$ of the total proteins. Another advantage of the ALFexpress DNA sequencer is the control of the gel temperature during electrophoresis. This possibility was used to characterize thermosensitive DNA-binding proteins. Band shift assays were carried out at different temperatures (30°C, 37°C, and 42°C) using the BlaITS mutant and the BlaIWT as references. These experiments showed that both

proteins gave similar results at 30°C. Increasing the temperature from 37°C to 42°C resulted in a decrease of bound DNA, counterbalanced by an equivalent increase of the free DNA (Figure 6). However, this effect was more important for BlaITS than for BlaIWT. Indeed, at 37°C, the percentages of bound DNA were equal to 37% and 71%, for BlaITS and BlaIWT, respectively. This ratio decreases to 18% for BlaITS at 42°C, whereas 53% of the BlaIWT-DNA complex remains detectable at this latter temperature (Figure 5).

Figure 3. Detection of multiple DNA-protein complexes. The Op1 + Op2_BI target (10^{-9} M) was incubated with increasing concentrations of BlaIWT (0 , 3.3×10^{-9} , 1.65×10^{-8} , and 3.3×10^{-8} M; panels A–D, respectively). Free: free DNA target; Cp1 and Cp2 are DNA-protein complexes corresponding to one and two occupied binding sites, respectively. The fluorescence is expressed in arbitrary units.

DISCUSSION

In this report, we demonstrate that an automated DNA sequencer can be used to analyze protein-DNA interactions and that all band shift assays described in the literature can be done with the help of this type of apparatus.

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The main advantages of this novel use of a DNA sequencer are the following: (i) the method is sensitive and 5 fmol oligonucleotides can be routinely detected; (ii) the fluorescent dye is chemically stable and is a good alternative to using radioisotopes; (iii) the temperature of the gel during the electrophoresis is controlled, and the method is suitable for studying the thermostability of DNA-binding proteins or to screen thermosensitive mutants; (iv) the method

is fast (120 min in most cases) and eliminates the time-consuming step of autoradiography; (v) the experimental data are saved directly onto the hard disk of the computer and analyzed easily with the help of analysis software.

Compared to a laser fluorescence scanner, Molecular Imager, or FluorImager, the ALFexpress DNA sequencer has a similar sensitivity (5 fmol) and gives the same results (data not shown). The total time of the experiment (i.e.,

gel electrophoresis and data acquisition) is faster when a Molecular Imager or a FluorImager is used with a short electrophoresis cassette ($10 \times 7.5 \times 0.5$ cm), and 1 h instead of 2 h for the ALFexpress. Nevertheless, a better resolution was achieved with the ALFexpress DNA sequencer. This result is caused by the longer migration of the sample through the gel. Indeed, to be detected, the sample must cross the laser beam at the bottom of the gel. With the ALFexpress DNA sequencer, a greater number of samples can be loaded onto the gel, and the temperature during gel electrophoresis can be controlled. Finally, with the addition of a cryostat, it allows for the acquisition of stable gel temperatures ranging from 15°C to 70°C and can be used to determine thermal transition curves.

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Figure 4. DNA binding assays with cellular extracts. In all cases, Free and Cp represent free DNA targets and DNA-protein complexes, respectively. The fluorescence is expressed in arbitrary units. *B. licheniformis* BlaI repressor is represented in panels A, B, and C. (A) DNA probe Op1_{BL} (0.5×10^{-8} M) was incubated with purified BlaIWT (3.7×10^{-7} M) or cellular extracts (125 μ g total proteins). The cellular extracts were prepared from *B. licheniformis* 749/I in presence (B) or absence (C) of the inducer. *C. freundii* AmpR repressor-activator is represented in panels D, E, and F. (D) DNA Probe Op_{CF} (0.5×10^{-8} M) was incubated with purified AmpR (6×10^{-7} M) or cellular extracts of *E. coli* TG1 harboring the pNU305 plasmid; 80 and 160 μ g total proteins are represented by panels E and F, respectively. In the AmpR band shift assays, Cp1 and Cp2 are probably the monomeric and dimeric AmpR bound to DNA, respectively (C. Jacobs, personal communication). In the purified AmpR (D), the purified protein was obtained from solubilized inclusion bodies and can no longer dimerize. In the data shown in panel E (80 μ g cytoplasmic proteins), 60% of bound AmpR is involved in the Cp2 complex. For 160 μ g cytoplasmic proteins (F), the Cp2 complex corresponds to 95% of bound AmpR.

Figure 5. Standard plot to estimate the quantity of BlaI in the cytoplasm of non-induced *B. licheniformis* cells. Various quantities of purified BlaIWT ranging from 2 to 8 ng were incubated with same concentration of Op1_{BL} target (0.5×10^{-8} M) and submitted to electrophoresis on a 4.5% non-denaturing polyacrylamide gel. The graph shows the ratio of the percentage of the bound Op1_{BL} target (BlaI-DNA complex) to the total Op1_{BL} target versus the quantity of BlaI. The arrow indicates the percentage of bound DNA present in 125 μ g cytoplasmic proteins of non-induced *B. licheniformis* cells.

Figure 6. Band shift assays to highlight the thermosensitivity of BlaIWT and BlaITS repressors. Before electrophoresis at 30°C, 37°C, or 42°C, the DNA-protein reaction mixtures containing 0.5×10^{-8} M of Op1_BI probe and 3.4×10^{-8} M BlaIWT or BlaITS were incubated at 30°C for 30 min. Free, free DNA target; Cp, DNA-protein. The fluorescence is expressed in arbitrary units.

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