J. Biochem. **138**, 473–478 (2005)
doi:10.1093/jb/mvi137

Exo-*Taq*-Based Detection of DNA-Binding Protein for Homogeneous and Microarray Format

Takashi Fukumori[1,2], Hirotaka Miyachi[1] and Kenji Yokoyama[1,*]

[1]Research Center of Advanced Bionics, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562; and [2]Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Ishikawa 923-1292

Received June 21, 2005; accepted July 27, 2005

The study of DNA–protein interactions is of great importance to understand basic cellular processes such as transcription, replication and recombination. In this research, we developed a novel detection system for DNA-binding proteins (DBPs) involving the exonuclease (Exo) III and *Taq* DNA polymerase reactions. The system consists of three steps, as follows: the target DBP in the sample solution is incubated with probe DNA, and the probe is digested with Exo III and then extended with *Taq* using fluorescent dye-labeled dUTP as a substrate. The DBP protects the probe from digestion by Exo III. Therefore, only the DBP-bound probe allows the following extension. We examined this system using the λ phage Cro repressor in a homogeneous format. The fluorescence image after gel electrophoresis showed a specific band. We also found that this system could be applied to the rapid and efficient detection of DBPs in stem and loop ds-DNA array formats. These results suggest that our method is useful as a new tool for analyzing DNA-protein interactions.

Key words: DNA-binding protein, DNA–protein interaction, exonuclease III stop assay, fluorescent dye-labeled nucleotide, *Taq* DNA polymerase.

Abbreviations: DBP, DNA-binding protein; Exo, exonuclease; *Taq*, *Taq* DNA polymerase.

Gene expression, which converts genetic information into functional proteins depends on various events including chromosomal activation and inactivation, and control of transcription initiation. In these events, the sequence-specific binding of many proteins, such as transcription factors, is critical for regulating gene expression. Furthermore, the specific binding of proteins to DNA is a key step for many cellular activities, such as DNA recombination and repair of DNA damage. Therefore, the detection of DNA-binding proteins (DBPs) as well as the identification of specific-binding sites is important to understand gene expression mechanisms and cellular functions. Additionally, the information obtained on the study of DNA-protein interactions can be applied to drug discovery and diagnosis.

The following conventional methods for analyzing DNA-protein interactions are currently used: electrophoretic mobility shift assay (EMSA, *1*), filter binding assay (*2*), DNA footprinting (*3*), ELISA (*4*), southwestern blotting (*5*), and reporter constructs in yeast (*6*). However, these methods are not suitable for high-throughput analysis, because of the time-consuming procedures and the requirement of antibodies against the target DBP.

Recently, DBP detection using microarray technology was reported (*7–13*). However, there were some drawbacks, *i.e.*, a decrease in binding activity due to direct protein labeling, and the requirement of many antibodies for many target DBPs.

*To whom correspondence should be addressed. Tel: +81-29-861-2987, Fax: +81-29-855-3833, E-mail: ke-yokoyama@aist.go.jp

To overcome these problems, we developed a novel DBP detection method what can be used for homogeneous and microarray format associated with two commonly used modification enzymes, namely exonuclease (Exo) III and *Taq* DNA polymerase (Exo-*Taq* reaction). The method involves a stem-loop probe DNA that contains the consensus-binding site of the target protein, 5–10 base pairs (bp) away from both the loop region and the blunt end (Fig. 1A). The probes were designed so as to incorporate fluorescent dye-labeled dUTPs (any deoxynucleotide and fluorophore can be used) to the 3′ end at every other base. First, a probe is incubated with a sample solution to form a DNA-protein complex (Fig. 1, B and C). After binding, the probe is digested with Exo III, followed by heat inactivation, and then sequentially extended with *Taq* using fluorescent dye-labeled dUTP as one of the four substrates. Exo III generally digests ds-DNA from the 3′-OH blunt or recessed end specifically. When the target protein binds to the probe (Fig. 1B), an Exo III attack starting from the 3′-OH blunt end stops short of the binding site, protecting the rest of the probe from further exonuclease attack. Therefore, the remaining 3′ side is used as a primer in the extension step and the probe is re-extended. The use of fluorescent dye-labeled dUTP in the extension step leads to the incorporation of fluorescent dye-labeled bases in the 3′ region of the probe to which the target protein is bound. Therefore, DBP can be detected as the fluorescence signal of probe DNA.

The detection in our assay depends on the inhibition of Exo III digestion of probe DNA by the probe-binding target protein, the same principle as for the Exo III stop assay

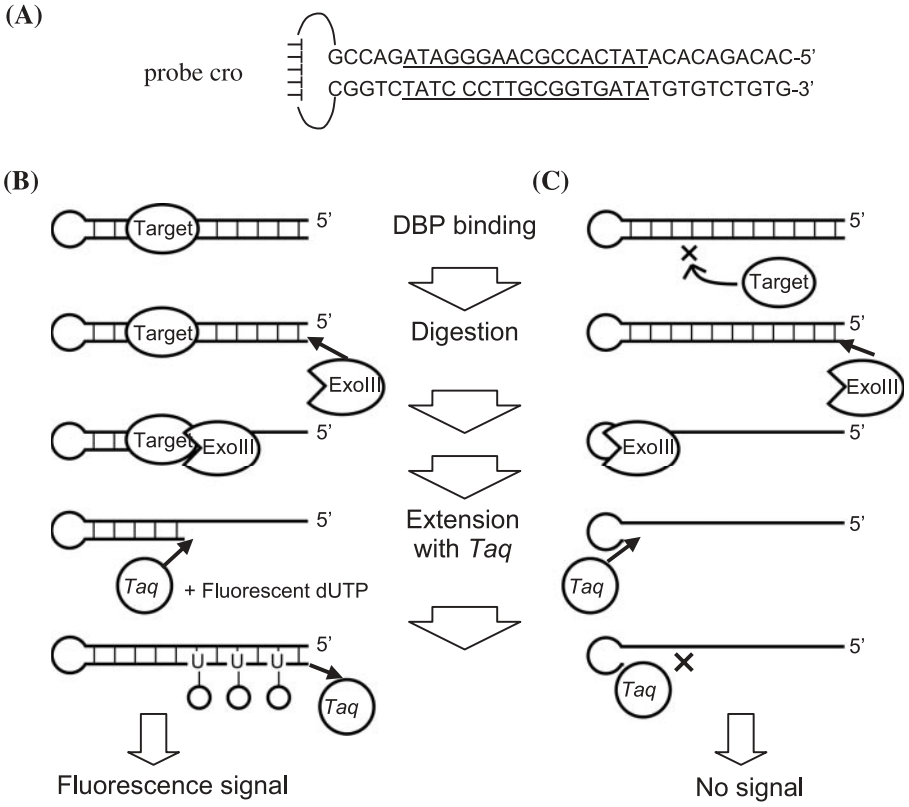

Fig. 1. **Schematic representation of the Exo-*Taq* reaction.** (A) Design of a stem-loop probe DNA. The stem-loop structure is formed by 32 nucleotides in the 5′ region and its reverse complement in the 3′ region with an internal TTTTT. (B) Probe containing the DBP-binding sequence. (C) Probe with an unrelated sequence. To detect target DBP, we first digest the probe with Exo III, followed by strand extension using *Taq*. When DBP binds to the probe for a given recognition sequence, the exonuclease activity would have intermediate stops; the site of an intermediate stop of the exonuclease activity on the probe serves as a primer for the subsequent strand extension. In contrast, if unrelated sequences are present, exonuclease will digest the probe until the edge of loop structure. Stronger fluorescence signals can be obtained when a probe containing DBP-recognition sequences in combination with fluorescent dye-labeled deoxynucleotides is used.

(*14–16*). The extension of DNA using thermostable *Taq* DNA polymerase, commonly used in PCR (*17*, *18*), is sufficiently rapid for the extension of the digested probe in our method (the extension is completed within 1 sec theoretically). The Exo-*Taq* reaction is a label-free protein assay and could be applied to the detection of many DBPs, therefore, this method would be suitable for use in a microarray format in terms of simultaneous detection or cost reduction. We applied the Exo-*Taq* reaction to microarray analysis. After the Exo-*Taq* reaction, fluorescent dye-labeled dUTP is incorporated covalently into the digested probe DNA sequence specifically on a glass slide, allowing sensitive detection with a hard washing procedure. In this paper, we describe experimental verification of the Exo-*Taq* reaction in a homogeneous format and its applicability to a microarray format.

Table 1. **Probe DNAs used in this study.**

Name	Sequence (5′→3′)
In homogeneous	
probe cro	CACAGACACATATCACCGCAAGGGATAGAC-CG-(5dT)-CGGTCTATCCCTTGCGGTGATATG-TGTCTGTG
probe p50	CACAGACACACACACGGGACTTTCCCAGAC-CG-(5dT)-CGGTCTGGGAAAGTCCCGTGTGT-GTGTCTGTG
In microarray	
probe cro-m	(C12-NH_2)–(10dT)-GAGACAGAGATATCACC-GCAAGGGATAGACCG-(5dT)-CGGTCTATCC-CTTGCGGTGATATCTCTGTCTC
probe cont.-m	(C12-NH_2)–(10dT)-GAGACAGAGACTCTCGG-GACTTTCCCAGACCG-(5dT)-CGGTCTGGGA-AAGTCCCGAGAGTCTCTGTCTC

MATERIALS AND METHODS

Preparation of Proteins—The gene encoding Cro was amplified from λ DNA by PCR by the introduction of *Bam*HI and *Eco*RI sites using the following primers: 5′-CGGGATCCATGGAACAACGCATAACCCT-3′ and 5′-GGGAATTCTTATGCTGTTGTTTTTTTG-TTACTC-3′. The amplified *cro* gene was cloned into expression vector pGEX-6P-2 (Amersham Biosciences), which encodes the recognition sequence for site-specific cleavage by PreScission Protease (Amersham Biosciences) located between the glutathione S-transferase (GST) domain and the multiple cloning sites. The GST fusion Cro was expressed in *Escherichia coli* BL21 (DE3)pLysS, bound to glutathione-Sepharose 4B resin (Amersham Biosciences), washed with phosphate-buffered saline, and then eluted with reduced glutathione. The GST moiety was cleaved with PreScission protease according to the manufacturer's (Amersham Biosciences) protocol. Purified NF-κB p50 was purchased from Promega.

Preparation of Probe DNAs—Probe DNAs were purchased from Tsukuba Oligo Service Co., Ltd. The probes used in this study are shown in Table 1. Before use, all probes in buffer (probes cro and p50, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 7.6; probe cro-m, 150 mM sodium phosphate, pH 9.0) were annealed at 95°C for 5 min and then allowed to cool gradually to room temperature.

Exo-Taq Reaction in a Homogeneous Format—The probe DNA was incubated with DBP at room temperature for 1 h

in a reaction buffer (10 mM Tris-HCl, 50 mM KCl, 3.0 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 ng ml^{-1} λ DNA, 0.5 mg ml^{-1} BSA, 0.05% NP-40, 10% glycerol, pH 7.6). The binding mixture was digested with Exo III (0.6 U μl^{-1}; Takara Bio Inc.) at 37°C for 10 min, followed by heat inactivation at 72°C for 5 min. Extension of the digestion mixture was sequentially performed by adding *Taq* (1 U; Takara Bio Inc.) and deoxynucleotides [final concentrations: 40 μM dATP, 40 μM dGTP, 40 μM dCTP, 40 μM Alexa Fluor 488-5-dUTP (Invitrogen)] at 72°C for 5 min. After inactivation by adding EDTA to the final concentration of 20 mM, the reaction mixture was purified on a Sepharose G-50 spin column (Amersham Biosciences) and resolved by 8% denaturing polyacrylamide gel (7 M urea, 25% formamide) electrophoresis for 15 min at 250 V. The gels were scanned with a FluoroImager 595 (Amersham Biosciences) with a voltage setting of 700 or 1,000 V and a scanning resolution of 200 μm. The band intensities were quantified with FragmeNT Analysis V4.2a (Amersham Biosciences). In the competition assay, competitor double-stranded (ds) DNA was added to the reaction buffer and then the Exo-*Taq* reaction was performed as described above.

Microarray Printing—Microarrays were printed on CodeLink Activated Slides (Amersham Biosciences) coated with a hydrophilic polymer containing *N*-hydroxysuccinimide ester groups. Amino-modified probe DNA (25 μM) in the printing buffer (150 mM sodium phosphate, pH 9.0) was spotted (25 nl) using a BIO DOT MS-7000 (Cartesian Technologies) at 45% relative humidity. Printed slides were incubated at room temperature overnight in a sealed chamber containing a saturated NaCl solution and residual reactive groups were blocked with a prewarmed blocking solution (50 mM ethanolamine, 0.1 M Tris-HCl, pH 9.0) at 50°C for 30 min, followed by rinsing with deionized water. These slides were washed with the washing buffer (4× SSC, 0.1% SDS) at 50°C for 30 min on a shaker, rinsed briefly with deionized water and then incubated in boiling water for 1 min, followed by rinsing with deionized water. The slides were then air dried and stored at room temperature until use.

Microarray Analysis—Prior to use, a printed slide was pre-incubated with the reaction buffer (10 mM Tris-HCl, 50 mM KCl, 3.0 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mg ml^{-1} BSA, 0.05% NP-40, 10% glycerol, pH 7.6) for 1 h at room temperature. The slide was incubated with the sample protein in the reaction buffer at room temperature for 1 h and then incubated sequentially at 37°C for 15 min. The Cro-bound slide was digested with Exo III (0.6 U μl^{-1}) at 37°C, followed by heat inactivation at 72°C and sequential extension with *Taq* (0.08 U μl^{-1}) in the extension buffer (10 mM Tris-HCl, 50 mM KCl, 3.0 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mg ml^{-1} BSA, 0.05% NP-40, 10% glycerol, 40 μM dATP, 40 μM dGTP, 40 μM dCTP, 40 μM, Alexa Fluor 488-5-dUTP, pH 7.6) at 72°C for 5 min. After the enzymatic reactions, the slide was washed with a washing buffer (4× SSC, 0.1% SDS) at 80°C for 1 min, rinsed briefly with deionized water and then air dried. The slide was scanned with ScanArray Express (PerkinElmer) at 10 μm resolution, 85% laser power, and 78% PMT gain. The signal intensities of the spots were quantified using ImageQuaNT V4.2a.

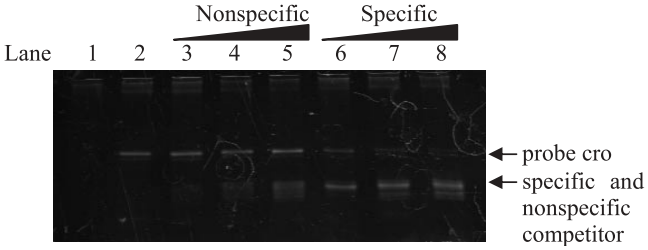

Fig. 2. **Detection of Cro using the Exo-*Taq* reaction.** Probe DNA (100 nM) was incubated with Cro (50 nM), and the DNA-protein complex was digested with Exo III and extended with *Taq*. Lane 1: Mixture of control probe (unrelated sequence) and Cro reacted with Exo III and *Taq*. Lane 2: Complex of probe cro and Cro reacted with Exo III and *Taq*. Lanes 3–5: Complex of probe cro and Cro reacted with Exo III and *Taq* in the presence of nonspecific competitor ds-DNA (lane 3, 100 nM; lane 4, 200 nM; lane 5, 500 nM). Lanes 6–8: Complex of probe cro and Cro reacted with Exo III and *Taq* in the presence of specific competitor ds-DNA (5′-CACAGACACATATCACCGCAAGGGATAGACCGCTGCA-3′, 3′-GTGTCTGTGTATAGTGGCGTTCCCTATCTGGCG-5′; lane 6, 100 nM; lane 7, 200 nM; lane 8, 500 nM). Upper band: specific fluorescence bands of probe cro; lower band: fluorescence bands resulting from specific and nonspecific competitors.

RESULTS AND DISCUSSION

Experimental Verification of the Exo-Taq *Reaction*—To confirm the accuracy of this method, we used the λ phage Cro repressor protein (Cro, *19*) as a well characterized DBP. Probe DNA containing the 17-bp operator OR3, which is a Cro consensus binding site, was placed 10-bp and 5-bp away from the blunt end and loop region, respectively. The probes were incubated with Cro, and then treated with Exo III and *Taq*. The reaction mixture was resolved by denaturing polyacrylamide gel electrophoresis. The resulting fluorescence image shows the specific fluorescence band for the mixture of the probe cro and Cro in lane 2, Fig. 2. This band clearly demonstrates fluorescent dye-labeled dUTP incorporation at the 3′ end of probe during the extension step. Note that the exonuclease attack stopped short of the Cro-binding site, allowing the undigested residual probe to function as a primer for further strand extension. In contrast, no specific band was observed in the case of Cro-binding site replacement with an unrelated sequence (lane 1). It seems that the probe was digested close to the loop region and the residual 3′ end was too short to act as a primer in the following extension, confirming the sequence specificity of this method. The competition assay was performed for further understanding of this method. Both specific (containing Cro-binding site) and nonspecific (unrelated sequence) competitor ds-DNAs were added to the reaction buffer, and then the Exo-*Taq* reaction was performed. The nonspecific competitor did not affect the intensity of the specific fluorescence band of the probe cro in the presence of Cro (lanes 3–5), whereas the specific competitor containing the Cro consensus sequence decreased the intensity of the specific fluorescence band (lanes 6–8). These results constitute further evidence for the specificity of this method. Figure 3 shows the calibration curve for the Cro protein. As the amount of Cro increased from 0 to 250 nM, the fluorescence intensity of the specific bands increased. This indicates that this

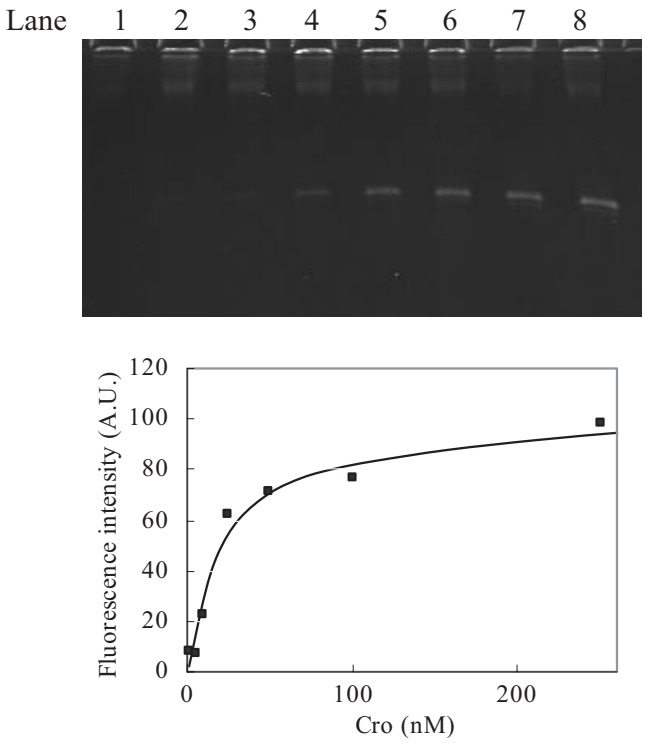

Fig. 3. **Correlation between the concentration of Cro and the fluorescence intensities of specific bands.** Exo-*Taq* reactions were performed using probe cro (100 nM) and the following amounts of Cro (nM): 0, 2, 5, 10, 25, 50, 100 and 250. The resulting fluorescent bands were quantified by Fragment Analysis. A.U., arbitrary units.

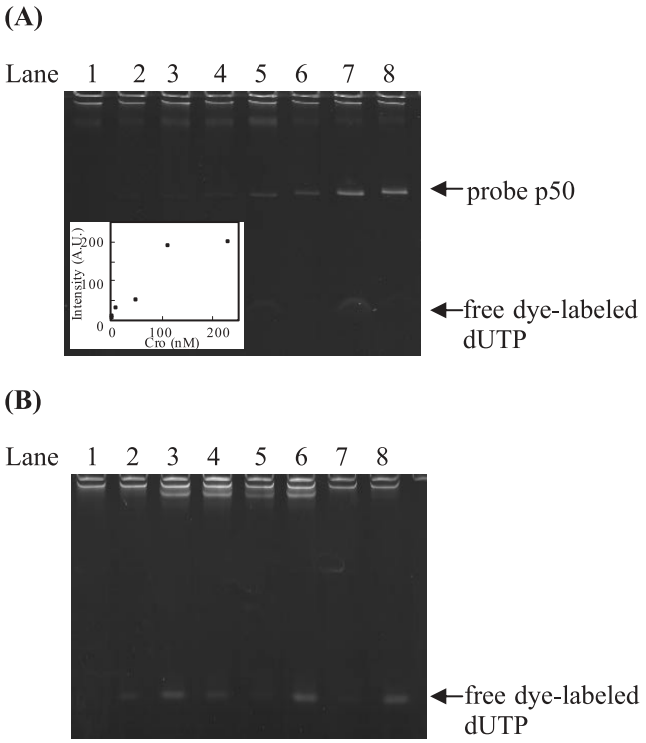

Fig. 4. **Detection of p50.** (A) Exo-*Taq* reactions were performed using probe p50 (100 nM) and the following amounts of p50 (nM): 0, 2, 5, 13, 25, 50, 115 and 230. Inset: Correlation between the concentration of NF-κB p50 and the fluorescence intensities of specific bands. A.U., arbitrary units. (B) Exo-*Taq* reactions were performed using an unrelated probe (100 nM) and the following amounts of p50 (nM): 0, 2, 5, 13, 25, 50, 115 and 230.

method can be applied to the quantification of DBPs. To confirm the general applicability of this method to other DBPs, we examined this method for transcription factor NF-κB p50 (*20*, *21*). We prepared a probe (probe p50) containing the p50-binding site, and then the Exo-*Taq* reaction was performed. The resulting fluorescence image shows the protein concentration-dependent band of the probe p50 (Fig. 4A), whereas the unrelated probe gave no specific bands (Fig. 4B).

Exo-Taq Reaction on a Solid Surface—We applied the Exo-*Taq* reaction to solid state microarray analysis. 5′-Amino modified (amino modifier C12) stem-loop probe DNA with a 10 dT DNA spacer at the 5′ end was immobilized covalently on a polyacrylamide-coated glass slide (Fig. 5A). The stem region consists of three parts; the central recognition site, a 5-bp flanking sequence proximal to the loop region, and the distal part at the 3′ end that alternately contains the T sequence for the incorporation of fluorescent dye-labeled dUTP in the extension step. To confirm the reactivity of *Taq* with the immobilized probe DNA, extension of the immobilized probe with fluorescent dye-labeled dUTP was performed (Supporting information 1, http://staff.aist.go.jp/ke-yokoyama/pdf/fukumori_suppinform.pdf). The fluorescence image revealed that the pre-digested probe from which 10 bases of the 3′ end were deleted showed a strong signal. In contrast, the non-digested probe, which did not incorporate fluorescent dye-labeled dUTP, showed almost no signals. These results suggest that stem-loop probe DNA is accessible to *Taq* and that fluorescent dye-labeled dUTPs can be incorporated at the 3′ end even when the probe is immobilized on a solid surface.

To investigate the reactivity of Exo III on a solid surface, probe DNA with dye-labeled dUTP incorporated was immobilized and digested with Exo III. As a result, the fluorescence intensity of the spot decreased by more than 98% within 10 min despite the steric hindrance of the fluorophore (Supporting information 2, http://staff.aist.go.jp/ke-yokoyama/pdf/fukumori_suppinform.pdf). In contrast, when the slide was washed under hard conditions (4× SSC, 0.1% SDS; 80°C, 1 min), the loss of fluorescence intensity was less than 5%. These results confirm that Exo III and *Taq* can react with the immobilized probe sufficiently, and that the hard washing procedure can be performed to eliminate unreacted fluorescent dye-labeled dUTP on the array surface, resulting in a high signal-to-noise ratio of the target protein.

Experimental Verification of DBP Detection on a Solid Surface—To verify the applicability of the Exo-*Taq* reaction to the microarray format, we evaluated the detection of Cro on a solid surface. Stem-loop probe DNA containing the Cro-binding site (probe cro-m, Fig. 5A) and an unrelated control probe (probe cont.-m) were immobilized on a polyacrylamide-coated slide. After incubation of Cro (from 0 to 200 nM) on the slide, the Exo-*Taq* reaction was performed, followed by washing with buffer. The relationship between the Cro concentration and fluorescence intensity is shown in Fig. 5C. The fluorescence intensity of

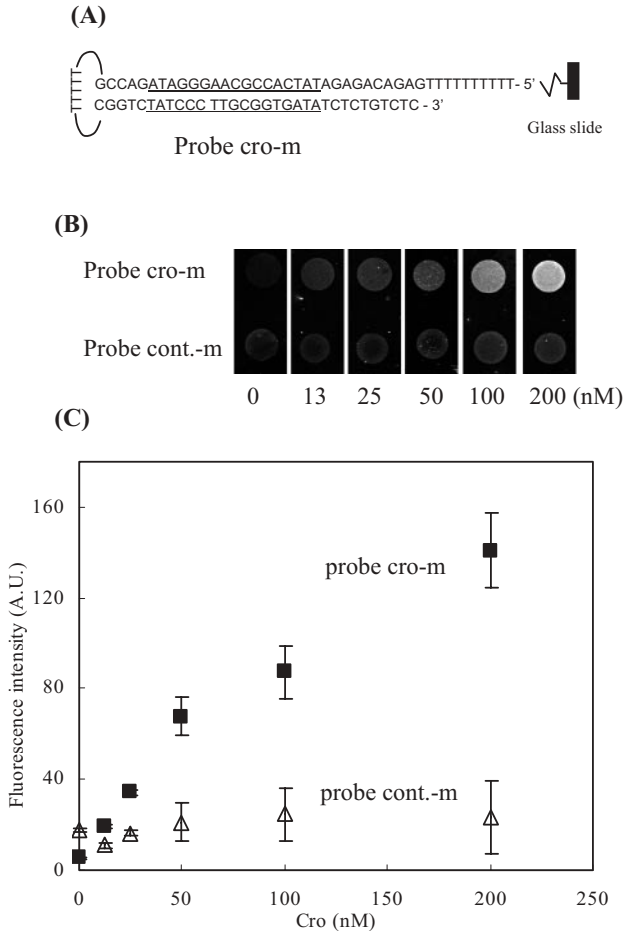

Fig. 5. **Exo-*Taq* reaction with various concentrations of target DBP on a solid surface.** (A) Structure of the probe cro-m on a glass surface. (B) Fluorescence images after the Exo-*Taq* reaction on a solid support. Probes cro-m and cont.-m (unrelated control sequence) were incubated with Cro (0, 13, 25, 50, 100, 200 nM) on a glass slide, followed by Exo III digestion, and then extended with *Taq*. (C) Correlation between the concentrations of Cro, and the fluorescence intensities of probe cro-m (closed symbols) and probe cont.-m (opened symbols). A.U., arbitrary units.

probe cro-m increased proportionally, whereas for probe cont.-m, there was no correlation between the DBP concentration and fluorescence intensity, suggesting that the Exo-*Taq* reaction can be performed on a solid surface and can be applied to the quantification of DBPs, as in the homogeneous format.

CONCLUSION

In this paper, we described a novel method for the detection of sequence-specific DBPs based on enzymatic reactions involving Exo III and *Taq*. The Exo-*Taq* reaction consists of very rapid and simple procedures; therefore, we can apply this reaction to various assay formats, including homogeneous and microarray ones. We first confirmed that this reaction can be used to analyze DBPs in a homogeneous format assay. Fluorescent dye-labeled dUTP was incorporated specifically into the probe DNA containing the Cro consensus site. Furthermore, our method has been applied to p50 as for Cro. The advantage of this method is that we can detect a target DBP without protein labeling, which could affect the binding activity of the target protein by changing the conformation of the protein. Furthermore, a fluorescent dye-labeled deoxynucleotide can be replaced by other labeled deoxynucleotides such as biotin- and digoxigenin-labeled nucleotides, depending on the detection method. We can also detect DBPs in combination with several other detection methods such as fluorescence polarization.

We applied the Exo-*Taq* reaction to the microarray format. Cro was detected using the Exo-*Taq* reaction on a solid substrate (Fig. 5). Fluorescent dye-labeled deoxynucleotides are incorporated covalently into the immobilized probe DNA through the Exo-*Taq* reaction, allowing hard washing without resulting signals being affected; therefore, the problem of nonspecific adsorption to the slide can be avoided.

Comparison with conventional methods, most well known methods for DBP detection, *i.e.*, EMSA (*1*) and DNA footprinting (*3*), rely on electrophoresis of probe DNA and require a radio-labeled probe for a sensitive assay. Thus, these assays are daylong events and the number of samples is limited. The filter binding assay (*2*) is also used as a sensitive assay for DBP, however, similar problems exist, *i.e.*, the use of a radio-labeled probe and time-consuming procedures. In contrast, the Exo-*Taq* reaction can be completed within 20 min in both the homogeneous and microarray formats. The sensitivity and dynamic range of our method in the homogeneous format is less than or comparable to those of EMSA (*22*). However, they could be further optimized by changing the probe design, i.e., increasing the number of sites for incorporation of labeled nucleotides or adding more than one binding site in both the homogeneous and microarray formats.

Recently, microarray-based methods (*7–13*) that are suitable for high-throughput assays were reported. The detection in these methods depends on antibodies to target DBP or direct protein labeling. In contrast, the detection in our method depends on the same principle as in the Exo III stop assay (*14–16*). Exo III activity is rather stable and the Exo III stop assay can be used for many DBPs. Our method could be applied not only to Cro but also to p50, which is a practical target for therapy for inflammation. Furthermore, our method is cheap, because it involves commonly used enzymes. The Exo-*Taq* reaction could be applied to many proteins with simple procedures and is far superior to antibody-based detection in the microarray format in terms of cost performance. In conclusion, we suggest that the detection method described here could be adapted for rapid and high-throughput analysis of DBPs.

We wish to thank Dr. S. Taira and Mr. K. Itoda for the helpful discussions.

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