Detection of Proteins Binding to the Promoter Region DNA Using a Nonradioactive Gel-Retardation Assay

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Binding of nuclear proteins to the promoter region was studied by a nonradioactive gel-retardation assay. The procedure uses biotinylated oligonucleotides in combination with streptavidin and biotin-conjugated alkaline phosphatase. This method offers sensitivity comparable to radioactive detection, and the advantage of the high stability of probes. Moreover the hazards of usage associated with radiation are avoided.

Key words: DNA-binding protein; nonradioactive; gel-retardation assay

The gel-retardation assay is a simple and rapid method for detection of sequence-specific DNA-binding proteins.1 This assay is based on the separation of free DNA fragments and DNA-protein complexes due to the differences in their electrophoretic mobilities in native polyacrylamide or agarose gels. The DNA fragments have conventionally been labeled with 32P and detected by autoradiography after the electrophoresis. In this report, we describe a nonradioactive method for the gel-retardation assay using a biotinylated probe. This assay system would make it easier to detect the DNA-binding activity of nuclear proteins without handling radioisotopes.

The promoter region of a rice gene for α-amylase, RAmy3D, was used. α-Amylases are important in the degradation of starch in various rice tissues,2,3 RAmy3D is highly expressed in rice suspension cells. Expression of RAmy3D gene in suspension cells is repressed at the transcriptional level by the presence of sugar in the suspension medium and induced by their absence.3,4

Nuclear proteins were prepared from 2-week-cultured rice suspension cells in which the RAmy3D gene was highly expressed. The procedure used was the same as previously described.5

The 358-bp of fragment (position −422 to −65), just upstream of TATA box was amplified by PCR using the RAmy3D forward (5′-biotin-TATGTGCATATAGACGG-3′) and reverse (5′-GTCACCGTGCGAGGGAGAT-3′) primers. PCR was done with 100 ng of a plasmid DNA, pOSg1.5S, in which the upstream region of the RAmy3D gene had been subcloned,6) 5 μl of 20 μM forward and reverse primers and 50 μl of the PCR pre-mix kit (Boehringer Mannheim, PCR master) in a final volume of 100 μl. The reaction commenced with 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 2 min at 55°C and 2 min at 72°C, ended with 10 min at 72°C.

Amplified products were labeled with biotin at the 5′-end of the coding strand for chemiluminescent detection. Products were size-fractionated on a 1.5% agarose gel and purified by using the standard electrophoresion procedure.7

The gel-retardation assays were done as described before.8

After electrophoresis, the biotinylated oligonucleotide and oligonucleotide-protein complexes were transferred to a nylon membrane (Immobilon-S) using a semi-dry blotter (Nihon Eido) at 100 mA for 30 min. A nylon membrane and filter paper (Whatman 3C) were incubated in blotting buffer (0.2 X TBE) for 30 min before use. The oligonucleotides were fixed to the nylon membrane by UV cross-linking (254 nm, 120 mJ/cm²) and the membrane was dried at 70°C for 10 min.

The membrane-bound probes were detected with streptavidine and biotinylated alkaline phosphatase using the chemiluminescent Lumigen-PPD as the substrate. The biotinylated probes were bound by streptavidine and followed by biotinylated alkaline phosphatase. The alkaline phosphatase was detected by autoradiography with the chemiluminescence produced during the enzymatic dephosphorylation of Lumigen-PPD. All the steps of the detection were done with a Phototope 6K Detection Kit (NewEngland BioLabs) in accordance with instructions. The chemiluminescent signals were recorded on an X-ray film by 30-min to 2-h exposures.

Figure 1a shows the PCR amplified products using the two RAmy3D primers. The predicted length of the reaction product was 358 bp, in which several functionally important sequences were involved, for example the pyrimidine box (C/T CTTTT C/T) and CATC box (TACCCAT).9 These elements are highly conserved sequences in the promoter regions of several cereal α-amylase genes.

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Abbreviations: PCR, polymerase chain reaction; TBE, Tris-borate/EDTA electrophoresis buffer.

Note
Nonradioactive Detection of DNA-Binding Proteins

Fig. 1. Amplification of Biotinylated Probes (358-bp) Using the RAmy3D Primers.

Plasmid pOSg1.5S in which the upstream region of RAmy3D had been subcloned was used for the template of PCR (a). Total rice genomic DNA prepared from young leaves by the standard CTAB procedure was used for PCR (b). Fragment sizes were estimated using the DNA molecular marker VI (Boehringer Mannheim). Arrowheads indicate the amplified fragment.

When 1 µg of rice genomic DNA was used as the template instead of the plasmid DNA, we also obtained the same product (Fig. 1b), indicating that these primers amplify the authentic RAmy3D promoter region from the total rice genomic DNA.

To establish conditions for the detection of the biotinylated probe on electro-transferred nylon membrane, a 20-µl sample of a reaction solution containing different concentrations of the probe without protein was resolved by electrophoresis on a polyacrylamide gels. The conditions of electrophoresis were the same as for the gel-retardation assay. The result indicates that 2 ng of biotinylated probe is enough for detection by the X-ray film (data not shown). The concentrations of the probe were measured by a gel-blot method, with a known concentration of herring sperm DNA as a standard.

Figure 2a demonstrates a typical nonradioactive gel-retardation assay using the 358-bp of RAmy3D promoter fragment. In the presence of nuclear protein, one major (B1) and one minor band (B2) with lower electrophoretic mobilities than the free probe were observed. When a 100-fold concentration of unlabeled RAmy3D promoter fragment was added to the binding mixture, both bands of DNA-protein complexes were specifically attenuated. The intensity of B1 was unaffected by the addition of the non-specific competitor, a digest of pBluescript KS, indicating that B1 represents a specific binding complex while B2 represents a non-specific binding complex.

When compared with the radioactive detection (Fig. 2b), this nonradioactive gel-retardation assay showed comparable signal intensities, and gave identical band patterns. These results demonstrate that this method has equivalent sensitivity to those by the radioactive procedure for detection of DNA-binding activity, and provide us precise information about the regulatory cis and trans-acting elements more easily.

One of the major problems of our nonradioactive detection is a high background caused by the non-specific binding of the streptavidine and/or biotinylated alkaline phosphatase to the nylon membrane. We found the background could be reduced somewhat by using highly purified probes, and/or reprobing with prolonged washing steps.

This nonradioactive gel-retardation assay system has some distinct advantages over the radioactive systems. Biotinylated probes are stable for prolonged periods, and the hazards associated with radioactive substances would be avoided. In addition to these advantages, biotinylated probes are widely applicable for several biochemical techniques. For example, the biotinylated oligonucleotides can easily be coupled with streptavidin immobilized agarose beads only by mixing. These beads can be used for DNA-affinity chromatography, which greatly facilitates purification of sequence-specific DNA-binding proteins. After the binding reaction in the gel-retardation assay described above, the assay mixtures are purified directly. The oligonucleotide-protein complexes are coupled with the streptavidin agarose beads, and the DNA-binding proteins are simply eluted with buffer containing an appropriate salt concentration. By using this technique, we have identified sequence-specific DNA-binding proteins for the promoter region of RAmy3D (S. Mitsunaga et al., unpublished results). Further studies will be aimed at identifying these proteins, which mediate the expression of RAmy3D.

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References


