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Rapid detection and purification of sequence specific DNA binding proteins using magnetic separation

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Abstract: In this paper, a method for the rapid identification and purification of sequence specific DNA binding proteins based on magnetic separation is presented. This method was applied to confirm the binding of the human recombinant USF1 protein to its putative binding site (E-box) within the human *SOX3* protomer. It has been shown that biotinylated DNA attached to streptavidin magnetic particles specifically binds the USF1 protein in the presence of competitor DNA. It has also been demonstrated that the protein could be successfully eluted from the beads, in high yield and with restored DNA binding activity. The advantage of these procedures is that they could be applied for the identification and purification of any high-affinity sequence-specific DNA binding protein with only minor modifications.

Keywords: biotinylated oligonucleotides, magnetic separation, protein–DNA interaction.

INTRODUCTION

Streptavidin coated magnetic particles are a solid-phase matrix for simple and efficient binding of biotinylated compounds, such as small molecules, peptides, proteins, antibodies, sugars, lectins, oligonucleotides, *etc.*[1] The affinity of biotin for streptavidin ($K_d = 10^{-15}$) is one of the strongest and most stable interactions in biology.[2] The biotin–streptavidin binding is resistant to high concentrations of salt and urea.[2] Magnetic particles allow the isolation and subsequent handling of target molecules in a highly specific manner. The separations and multiple washes or rounds of purification can be completed rapidly.

Biotinylated oligonucleotides are now a well-established and very useful tool in many fields of biological research, such as nucleic acid hybridisation, protein purification and DNA–protein binding studes.[3–5] The oligonucleotide pull down assay is a widely used technique for the identification and purification of sequence specific DNA binding proteins.[5,6] The DNA fragment endlabeled with biotin is attached to the streptavidin coated magnetic particles. Incubation with a protein frac-

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tion enables the sequence specific DNA binding protein to bind to the particles. By placing a strong magnet against the wall of the tube, the magnetic particles with the specific protein attached are separated from any contaminants remaining in the solution. After a few washes, the bound proteins can be analysed by Western blot.[7]

In previous studies, the potential role of USF1 protein (upstream stimulating factor 1) in the basal activation of the human *SOX3* gene was investigated.[8] USF1 is a transcription factor which preferentially interacts with the E-box (5'-CANNTG-3') DNA domain.[9,10] Supershift analysis showed the presence of USF1 in the protein complex bound to the *SOX3* promoter. The aim of this study was to confirm the direct binding of USF1 protein to its putative binding site (E-box) in the human *SOX3* promoter using the oligonucleotide pull down assay. The results showed that the human recombinant USF1 protein binds directly to the oligonucleotide containing the putative binding site within the human *SOX3* promoter attached to the streptavidin coated magnetic particles. It was also shown that the USF1 protein could be easily eluted from the magnetic beads in a high yield. The main advantage of this procedure is that the eluted protein restored its binding activity, and could be used in immunoreactions and DNA–protein binding studies.

EXPERIMENTAL

Bacterial strain and plasmid DNA

The human GST-USF1 expression vector (pGEX-2TK) was a kind gift from Dr. Benoit Viollet (Institut Cochin de Génétique Moléculaire, Université René Descartes, Paris, France). The *E. coli* strain BL21, pLysS[11] was transformed with the pGEX-2TK-USF1 construct.

Oligonucleotides

Following sense oligonucleotide, which contains an E-box element from the human *SOX3* promoter (5'- GAACCTGTCAATCACGGGTCCTCCGGGTTGC -3'), was annealed with the corresponding antisense oligonucleotide (5'- CAACCCGGAGGACCCTGGATTGACAGGTTC -3') in order to form a double standed DNA probe containing the consensus binding site for USF1.[10] For the oligonucleotide pull down assay, the antisense oligonucleotide was 5'-biotin labelled and the annealing reaction was carried out as described above.

Antibodies

Rabbit polyclonal antisera against USF1 was a kind gift from Dr. Benoit Viollet.

Protein purification procedures

GST fusion protein was expressed in *E. coli* (BL21, pLysS) and purified using the Glutathione *S*-transferase (GST) Gene Fusion System (Amersham Pharmacia Biotech). Briefly, the bacteria were transformed with recombinant pGEX-2TK plasmids expressing the GST-USF1 fusion protein. The overnight in ampicillin (250 μg/ml) grown bacterial culture was diluted (1:100) into 250 ml of Luria broth.[12] The growth was continued at 37 ºC to an A_{600} = 1.0, followed by induction with 1 mM isopropylthiogalactopyranoside for 60 min at 37 ºC. The cells were pelleted and resuspended in 5 ml of buffer A (50 mM KCl, 25 mM HEPES, pH 7.9, 6 % glycerol, 5 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05 % Triton X-100). The cells were lysed on ice by sonication and centrifuged at 12,000 × g for 10 min at 4 ºC. The supernatant was mixed for 1 h at 4 ºC on a rotator with 0.5 – 1 ml of 50 % glutathione-Sepharose beads (Amersham Pharmacia Biotech) that had been preswollen in buffer A. After absorption, the beads were collected by centrifugation at 4 ºC and washed three

times with 1 ml of buffer A. The fusion protein was recovered from the matrix using elution buffer (10 mM glutathione). The protein concentrations were determined by the method of Bradford.[13]

Preparation of magnetic DNA affinity beads

The increasing amounts (0.005; 0.01; 0.015; 0.02 mg) of streptavidin magnetic beads (Streptavidin MagneSphere Paramagnetic Particles, Promega) were washed twice with 0.5×SSC. Biotinylated oligonucleotide (100 ng) was bound to the beads in 1×TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and placed in a roller at room temperature for 30 min. The efficiency of coupling was visualized on a silver-stained 20 % non-denaturating polyacrylamide gel.[12]

Oligonucleotide pull down assay

GST-USF fusion protein (600 ng) was mixed with non-specific competitor DNA (1 μg of poly dIdC) in buffer A (20 mM Tris-HCl pH 8.0, 150 mM KCl, 1 mM EDTA, 15 % glycerol and 0.05 % Nonidet P-40).[14] Magnetic beads (0.02 mg) coupled with 100 ng of biotinylated oligonucleotide were added to the mixture. The non-specific adsorption was estimated in the same reaction using magnetic beads with no DNA adsorbed. The mixtures were held in the roller at room temperature for 30 min, followed by three washes with buffer A containing 75 mM KCl. The bound proteins were analysed using Western blot.[7]

Elution of recombinant USF1 protein from the DNA affinity beads

Oligonucleotide pull down reactions were carried out as described above using 500 ng, 1 μg and 1.5 μg of recombinant USF1 protein. After incubation and magnetic separation, the beads were washed three times with buffer A containing 75 mM KCl, and the fusion protein was eluted from the beads with buffer A containing 1 M KCl. Bovine serum albumin (0.5 μg/μl) was added to the washing and elution buffer. The yield of eluted proteins was analysed by Western blot and their ability to bind DNA by radioactive gel retardation assay.

SDS-PAGE and Western blot analysis

The protein samples and molecular weight standards (Fermentas) were separated by SDS-PAGE in 10 % resolving gel using a Bio-Rad minigel apparatus and then electrotransferred to a nitrocellulose membrane (Amersham-Pharmacia Biotech). The membrane was subjected to Western blot analysis[15] using polyclonal rabbit antisera against human USF1 and then donkey anti-rabbit peroxidase-linked IgG (Amersham Biosciences) as a secondary antibody. The immunoreactive bands were detected using ECL detection reagents, as described by the manufacturer (Amersham Biosciences).

Electrophoretic mobility shift assays (EMSA)

For the electrophoretic mobility shift assay, the annealed oligonucleotides were labelled by filling in the overhanging 5' ends using the Klenow fragment *E. coli* DNA polymerase (USB) in the presence of [α-^{32}P]dCTP.

The indicated amounts (see Figure 3 legend) of eluted fusion protein USF1 were incubated with 1 ng of ^{32}P-labeled oligonucleotide in binding buffer containing 20 mM Hepes pH 7.6, 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 5 mM DTT, 6 % glycerol and 1 μg of poly(dI-dC) in a total volume of 20 μl. The incubation was performed at 37 ºC for 30 min. The DNA–protein complexes were resolved on a 6 % non-denaturating polyacrylamide gel. The gel was dried and subjected to autoradiography at –70 ºC.

RESULTS AND DISCUSSION

Oligonucleotide pull down assay

A specific double stranded DNA fragment, 31 bp in length containing an E-box element from human *SOX3* promoter, was produced by annealing sense and antisense 5'-biotin labelled oligonucleotides. The biotin endlabeled fragment was

bound to streptavidin coated magnetic particles, to give magnetic DNA affinity beads having a high binding capacity. DNA was adsorbed to the surface of the particles, giving an advantage for the kinetics of protein binding.

To investigate the binding capacity of the streptavidin magnetic particles, double stranded biotinylated oligo was incubated with various amounts of beads (Fig. 1). The binding efficiency was analysed by polyacrylamide gel electrophoresis and the silver-stained gel was subjected to densitometric analysis. This analysis showed that 90 % binding efficiency was achieved using 0.015 and 0.02 mg of beads, while lower amounts (0.005 and 0.01 mg) were shown to be insufficient (50 % and 73 % efficiency, respectively). Therefore, it was decided to use 0.02 mg of streptavidin magnetic particles in the oligonucleotide pull down assay, ensuring that the majority of the supplied biotinylated oligo would be captured by the beads.

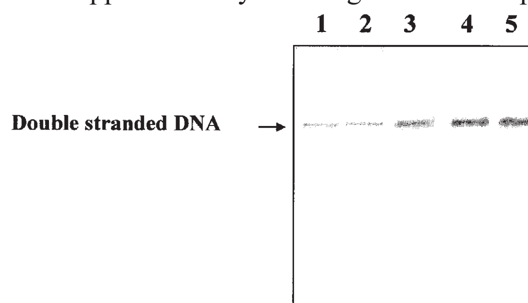

Fig. 1. Binding of biotinylated oligo to streptavidin magnetic particles. 100 ng of biotinylated oligo were incubated with increasing amounts (0.005, 0.01, 0.015, 0.02 mg, lanes 1–4, respectively) of streptavidin magnetic beads for 30 min. In the control reaction, 100 ng of biotinylated oligo was run on the same gel (lane 5). The binding efficiency was measured by densitometric analysis of the silver stained polyacrylamide gel.

In the oligonucleotide pull down assay, the biotinylated oligo bound to the streptavidin magnetic particles was incubated with recombinant human USF1 protein. A large excess of non-specific DNA (1 μg of poly dIdC) was added to reduce the potential, non-specific binding of the protein to the beads. In the control reaction, the same amount of the protein was incubated with magnetic beads with no DNA adsorbed. After incubation, magnetic separation and intensive washing, the beads were subjected to SDS-PAGE. The 65 kD protein corresponding to the GST fused USF1 protein was identified by Western blot analysis using anti USF1 antibody (Fig. 2). The human recombinant USF1 protein was detected only in the streptavidin magnetic beads incubated with biotinylated oligo (Fig. 2, lane 2), whereas no protein was observed in the control reaction (Fig. 2, lane 3). These results demonstrated that USF1 binds directly to its putative sequence (E-box) within the promoter of the human *SOX3* gene.

Elution of recombinant USF1 protein

The purpose of this study was to develop a techinique which could be applied for the identification of any sequence specific DNA binding protein. For this rea-

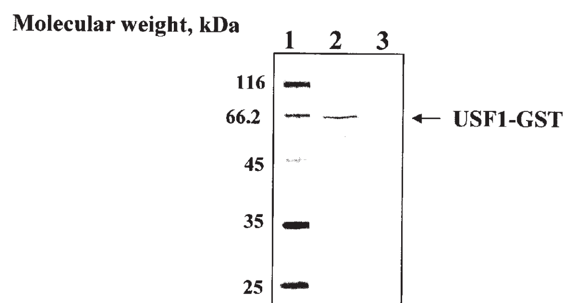

Fig. 2. Oligonucleotide pull down assay of the human recombinant USF1 protein. Biotinylated oligo (100 ng) bound to the streptavidin particles (0.02 mg) was incubated with recombinant USF1 protein (500 ng) for 30 min. The collected particles were run on a polyacrylamide gel, electrotransferred to a nitrocellulose membrane and subjected to Western blot analysis using an antibody against USF1 (lane 2). In the control (lane 3), protein incubated with beads (no DNA absorbed) showed no immunoreaction. The Ponceau S stain of molecular weight markers is presented in lane 1.

son, the conditions for protein elution from the magnetic particles were optimised and its DNA binding activity tested.

After incubation with protein and magnetic separation, the particles were washed with a buffer of intermediate ionic strength (75 mM KCl). The specifically bound protein was eluted by resuspending the beads in a buffer of higher ionic strength (1 M KCl). It was previously shown that bovine serum albumin acts as a stabilizer in the washing and elution steps, increasing the yield of eluted proteins.[14] Therefore, bovine serum albumin was added to both the washing and elution buffers at the same concentration (0.5 μg/μl).

The yield of USF1 protein eluted from the beads was analysed using Western blot (Fig. 3A). As a control, the same amounts of recombinant, non-treated, human USF1 protein were used in the immunoreactions. Thus, by using anti USF1 antibody, the recombinant USF1 protein was identified in all three eluted samples (Fig. 3A). Based on densitometric analysis and by comparison with the controls, the yields of the eluted proteins were estimated to be 70 % (Fig. 3, comparing lanes 2–4, with lanes 5–7). If the yield was not satisfactory after one adsorption, the method is rapid enough to be repeated in several cycles.

Electrophoretic mobility shift assays (EMSA)

To examine the DNA binding ability of the eluted USF1 protein, electrophoretic mobility shift assay was performed using 1 μg and 1.5 μg of eluted proteins with the same oligomer, now labelled with ^{32}P. As shown in Fig. 3B, band shifts were observed with both amounts of the eluted protein samples (Fig. 3B, lanes 2 and 4). The intensity of the DNA–protein complex increased with increasing amount of protein added to the binding reaction (Fig. 3B, lanes 2 and 4). The intensity of these complexes were similar to those obtained using the same amounts of recombinant protein (Fig. 3B, comparing lanes 2 and 3, 4 and 5). Thus, the results

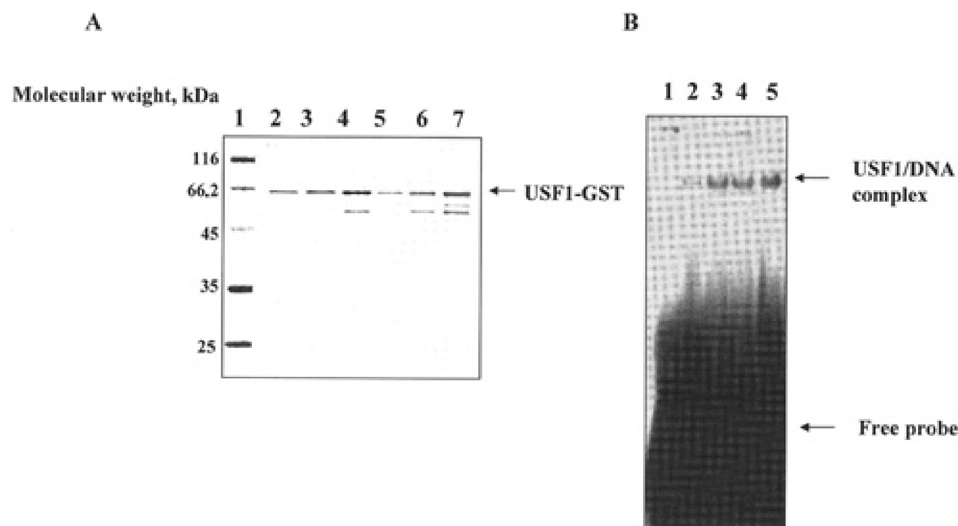

Fig. 3. Yield and binding activity of eluted proteins. (A) Elution of recombinant USF1 protein from the beads after oligonucleotide pull down assay. Biotinylated oligos bound to the streptavidin particles were incubated with 500 ng, 1 μg and 1.5 μg of recombinant USF1 protein for 30 min. The proteins were eluted from the collected particles and analysed by Western blot using an antibody against USF1 (lanes 1–3, respectively). In the controls (lanes 5–7, respectively) 500 ng, 1 μg and 1.5 μg of non-treated, recombinant USF1 protein were run on the same gel. The Ponceau S stain of molecular weight markers is ipresented in lane 1. (B) DNA-binding activity of eluted recombinant USF1 protein. Eluted USF1 protein samples (1 μg and 1.5 μg) were analysed by electrophoretic mobility shift assay using ^{32}P-labeled oligo containing an E-box element from human *SOX3* promoter (lanes 2 and 4). The same amounts of non-treated, recombinant USF1 protein were used in the control reactions (lanes 3 and 5). The free probe is shown in lane 1.

of this analysis showed that the DNA binding activity of the recombinant human USF1 protein eluted from the beads using a high-salt buffer was restored. Densitometric analysis of the shifted bands in the electrophoretic mobility shift assay showed that the recovered DNA binding activity was 70 %.

In conclusion, it should be pointed out that magnetic particles are ideally suited for applications involving high-throughput proteomic screening, small-scale protein isolation, immunomagnetic isolations or cell separation experiments. With magnetic beads, affinity purification of tagged proteins, antigens, antibodies and nucleic acids can be performed conveniently and rapidly, with minimal time necessary for the separation of the solid-phase from the solution. In addition, in this study it was demonstrated that the immobilized substrates remain biologically active and can be eluted in small volumes or serve as ligands in subsequent pull-down or target interaction experiments involving DNA or proteins.

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ИЗВОД

ПРИМЕНА МАГНЕТНОГ РАЗДВАЈАЊА ЗА БРЗУ ДЕТЕКЦИЈУ И ПРЕЧИШЋАВАЊЕ ПРОТЕИНА КОЈИ СЕ ВЕЗУЈУ ЗА СПЕЦИФИЧНЕ СЕКВЕНЦЕ НА ДНК

МАРИЈА МОЈСИН, ЈЕЛЕНА ЂУРОВИЋ, ИСИДОРА ПЕТРОВИЋ, АЛЕКСАНДАР КРСТИЋ, ДАНИЈЕЛА ДРАКУЛИЋ, ТИЈАНА САВИЋ и МИЛЕНА СТЕВАНОВИЋ

Инсшишуш за молекуларну генешику и генешичко инжењерсшво, Војводе Сшеие 444а, и. иp. 23, 11010 Београд

У овом раду је приказана метода за брзу изолацију и идентификацију протеина који се везују за специфичне секвенце на ДНК, заснована на магнетном раздвајању. Овом методом је потврђено директно везивање хуманог рекомбинантног протеина USF1 за потенцијално везивно место (Е блок) у промоторском региону хуманог *SOX3* гена. Показано је да се рекомбинантни USF1 протеин, у присуству компетиторске ДНК, специфично везује за ДНК фрагмент који је обележен биотином и везан за магнетне куглице обложене стрептавидином. Такође, показано је да се протеин може елуирати са куглица у високом приносу и са очуваним афинитетом за везивање за ДНК. Предност ове методе је што се, уз мање модификације, може успешно применити за преичишћавање и идентификацију свих протеина који се везују за специфичне секвенце на ДНК.

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