A new affinity purification procedure for DNA-binding proteins using bromoacetyl agarose

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Received March 23, 1995; Revised and Accepted May 10, 1995

Characterization of proteins binding to the promoters of eukaryotic genes has proved essential for understanding the transcriptional regulation of viral and cellular genes. Because of the low abundance of these proteins in the cell, conventional purification of these molecules has been laborious. In contrast, sequence-specific DNA affinity chromatography has greatly facilitated rapid isolation and characterization of DNA-binding proteins.

A number of procedures for coupling specific DNA sequences to resins have been reported to be effective (1–5). However, all have restrictions on the form of DNA which can be attached to the matrix. In case of avidin or streptavidin columns, biotin must be coupled to the DNA (1,2). Cyanogen bromide activated coupling requires single-stranded overhangs (3). A more recent technique by Larson and Verdine allows efficient coupling of blunt-ended double-stranded ODNs, but requires the use of complex modified nucleosides during DNA synthesis (4).

In most laboratories, a variety of double-stranded probes are available from gel shift analyses. It would be highly desirable to have a method which allows direct use of these double-stranded DNA probes for preparation of protein affinity purification columns. Recently, fast and efficient chemical autoligation in aqueous media between short oligomers terminated by thiophosphoryl and bromoacetamido groups has been reported (6,7). Here we describe the use of this chemistry for the simple, rapid and efficient attachment of any double-stranded DNA to a solid support.

The DNA loading procedure consists of the following two steps: (i) thiophosphorylation of a double-stranded DNA with γ -thio-ATP to generate a 5'-thiophosphoryl group and (ii) coupling of the thiophosphorylated DNA to bromoacetyl agarose resulting in efficient attachment of DNA to the resin. The derivatized resin was then used for the DNA affinity protein isolation. The scheme is shown as follows:

Scheme 1.

We have used this method to purify DNA-binding proteins from both bacteria and eukaryotic cellular extracts. The bacterial protein which was purified was the liver transcriptional activator protein (LAP), a member of the C/EBP β family identical to rat NF-IL6 (8). The expression clone (a partial *NcoI-SmaI* DNA fragment containing the LAP ORF which was inserted into pET 8c) was used to transform the bacterial strain BL 21/DE-3/pLysS (8). Extracts were prepared from bacteria grown for 3 h in the presence of 0.5 mM IPTG. The crude bacterial extracts were partially purified by salt gradient fractionation (0.2–1.0 M KCl) on heparin-agarose columns (Pharmacia LKB, Uppsala, Sweden) and then the eluted fraction, verified by gel shift, was run through the DNA affinity column prepared by the described method.

The DNA affinity column was generated from activated bromoacetyl agarose (Calbiochem, La Jolla, CA) coupled to double-stranded NF-IL6 probe. The sequence used for coupling was a trimer of the 14mer duplex:

(AGATTGTGCAATCT)
(TCTAACACGTTAGA)₃

Either the antisense or sense strand (200 nmol) was thiophosphorylated by T4 kinase (Promega, Madison, WI) in the presence of γ -thio-ATP, mixed with a known ratio of [γ -³⁵S]ATP (Amersham, IL), and unlabeled adenosine-5'-O-3-thiotriphosphate (Boehringer Mannheim, Germany). After thiophosphorylation of one strand, the complementary strand was annealed at 90°C for 5 min followed by cooling to room temperature in the presence of 250 mM Tris-Cl (pH 7.6), 100 mM NaCl and 1.5 mM MgCl₂. Then the double-stranded DNA probe was passed through a G-50 Sephadex column (Sigma) three times to remove the unreacted thiophosphates. Bromoacetyl agarose (1 ml) was washed in 2 ml of 100 mM sodium phosphate buffer (pH 7.0) for 1 h, and then incubated with the probe in 100 mM sodium phosphate buffer (pH 7.8) in a final volume of 1.5 ml, at room temperature, with shaking for a minimum of 4–16 h.

To block the remaining unreacted bromoacetyl groups on the resin, the affinity matrix was then incubated with a 50–100 molar excess of cold adenosine 5'-O-3 thiotriphosphate in 100 mM sodium phosphate buffer (pH 7.8) at room temperature for 1 h and then packed into a BioRad Poly Prep chromatography column.

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We have subsequently found that 100 mol excess of sodium thiophosphate (Sigma) in 100 mM sodium phosphate buffer (pH 7.8) also blocks efficiently and is more economical. The 5'-thiophosphorylation and coupling efficiency were both estimated from the associated radioactive counts, which were 70 and ~60%, respectively. The DNA affinity column was washed with 10 vol of buffer D (10 mM HEPES pH 7.9, 100 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol) and 10 vol of the same buffer containing 1 M KCl prior to re-equilibration in buffer D containing 100 mM KCl.

NF-IL6-specific gel shift binding activity was pre-enriched by recovery from the 400 mM KCl fraction of heparin-agarose loaded with crude bacterial extracts. This was pre-mixed with the non-specific competitor, polydI:dC, and then applied to the DNA affinity column by gravity flow. After loading, the resin was washed three times with 10 ml of 100 mM KCl in buffer D, and proteins were eluted by step gradients of 0.2–1.0 M KCl in 1 ml buffer D. Efficiency of the affinity protein purification and its DNA-binding activities were monitored by analysis of each eluted fractions using Coomassie blue staining, EMSA gel shift assay, Western blotting with a C/EBP β antibody and Southwestern blotting.

Using this method, NF-IL6 was eluted in the 600 mM KCl fraction from the affinity column, purified 100–150-fold from the heparin-agarose fractions (overall purification ~500-fold), yielding >95% purity from crude bacterial extracts (Fig. 1). The coupling efficiency of DNA to bromoacetyl resin was 50–60% with a column capacity of 70–80 nmol ODN/ml resin. This is higher than that achieved by conventional methods using cyanogen-bromide activation (3). This approach was also successfully used to purify CACCC binding proteins from nuclear extracts of tax transformed cells (Kang, S. H., Xu, X., Heidenreich, O. and Nerenberg, M., manuscript in preparation).

The advantages of this method compared with those previously described are (i) facile use of any double-stranded DNA without overhangs, (ii) no ligation is needed between the probe and the resin because the thiophosphate terminated DNA and the bromoacetyl agarose will chemically autoligate fast and efficiently at pH 7.5–8.5, and (iii) high coupling efficiency and capacity of the affinity column.

This method proved to be very rapid, specific and efficient to purify sequence-specific DNA-binding proteins from bacterial and eukaryotic cellular extracts which can be easily used with any double-stranded DNA probe.

ACKNOWLEDGEMENTS

The authors thank Dr Mario Chojkier for providing LAP expression vector. The work was supported by NS 12428 from NIH and a grant from Lynx Therapeutics to M.N.

Figure 1. (A) Denaturing 10% SDS-PAGE gel stained with Coomassie brilliant blue stain. M: 10 μ l marker proteins (BioRad, Hercules, CA, USA); lane 1: crude bacterial extract 10 μ g; lane 2: 2.0 μ g of heparin-agarose 400 mM KCl eluted protein; lane 3: 2.0 μ g of affinity column flow through; lane 4: 0.2 μ g of purified LAP (34 kDa) from 600 mM KCl elution of affinity column indicated by arrow. (B) Southwestern blot probed with NF-IL6 probe. Lane 1: crude bacterial extract 10 μ g; lane 2: heparin-agarose 400 mM KCl eluted protein 2 μ g; lane 3: affinity column flow through 2 μ g; lane 4: 0.2 μ g of purified LAP from 600 mM KCl elution of affinity column. (C) Electrophoretic mobility shift assay (EMSA) analysis using NF-IL6 as a probe. Samples were analyzed in a 5% non-denaturing polyacrylamide gel using 0.5 $\times$ TBE buffer (25 mM Tris, 25 mM boric acid, 1 mM EDTA, pH 8.3) as running buffer. Lanes 1–4 are the same as described in (B). F: free probe.

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