Short Technical Reports

Extraction of Nuclear Proteins with Increased DNA Binding Activity

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ABSTRACT

We have developed a method for the rapid extraction of nuclear proteins from cultured cells. The ammonium sulfate method described here extracts larger quantities of proteins that retain DNA binding activity than the modified Dignam method and another popular method used for the extraction of transcription factors. The ammonium sulfate method is rapid and can be used to process a large number of samples for gel shift analysis.

INTRODUCTION

Electrophoretic mobility shift assays (EMSA) assess the ability of DNA binding proteins to bind to specific nucleotide sequences. The specificity of DNA-protein interactions makes EMSA an invaluable tool for following the expression of transcription factors through various cellular events such as cell growth, growth arrest, apoptosis or differentiation. While working with stably transfected cell lines, our laboratory sought a rapid and easy method to produce nuclear extracts from many samples in a relatively short amount of time for use in gel shift analysis. We sought to minimize not only the labor involved but also the number of cells required to produce a sufficient amount of nuclear extract for gel shift analysis.

The first protocol to isolate soluble nuclear proteins was developed by Dignam et al. and involved the isolation of a crude nuclear fraction from large cell cultures, subsequent high-salt extraction in a buffer containing 420 mM NaCl and a lengthy dialysis step (2). Andrews et al. modified this protocol for use with much fewer cells and eliminated the need for dialysis (1). These modifications made it possible to use the modified Dignam procedure for the isolation of DNA binding proteins from

Table 1. Ammonium Sulfate Nuclear Extract Protocol

Step 1. Remove cells in wash buffer ^a . Pellet cells and discard supernatant. Perform steps 2–6 at 4°C.
Step 2. Resuspend cell pellet in 800 µL polyamine buffer ^b . Add 8 µL 10% NP-40. Leave on ice for 10 min.
Step 3. Centrifuge at 16 000× <i>g</i> for 2–5 min to pellet crude nuclei. Discard supernatant.
Step 4. Resuspend nuclei in 600 µL polyamine buffer. Underlay with 300 µL 30% sucrose in polyamine buffer. Centrifuge at 1300× <i>g</i> for 10 min.
Step 5. Remove supernatant. Resuspend nuclei in 3–4 volumes (40–80 µL) nuclear storage buffer ^c . Add 0.8 M AS equal in volume to the nuclear slurry. Vortex mix briefly. Leave on ice for 15–20 min.
Step 6. Centrifuge at 16 000× <i>g</i> for 5 min. Spin-dialyze supernatant through a 0.6-mL Biogel® P10 spin column (Bio Rad Laboratories, Hercules, CA, USA) equilibrated in P10 buffer ^d . Aliquot and flash freeze extracts.

^aWash buffer: 10 mM Tris-HCl (pH 8.0), 140 mM NaCl and 1 mM EDTA.

^bPolyamine buffer: 10 mM HEPES (pH 7.9), 10 mM KCl, 750 µM spermidine, 150 µM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 20 µg/mL PMSF, 0.5 µg/mL leupeptin, 1 µg/mL pepstatin, 50 µg/mL chymostatin, 1 µg/mL aprotinin and 50 µg/mL antipain.

^cNuclear storage buffer: 50% glycerol, 20 mM Tris (pH 8.0), 75 mM NaCl and 0.5 mM EDTA.

^dP10 buffer: 20% glycerol, 20 mM HEPES (pH 7.9), 60 mM KCl and 0.5 mM EDTA.

many different samples simultaneously. The Dignam procedure has since become the method of choice for many researchers studying transcription factors purified from cultured cells. An additional method for the purification of basic leucine zipper or bzip nuclear proteins was developed by Lavery and Schibler and is used for the purification of proteins from tissues such as rat liver (4). This method involves the purification of nuclei and the extraction of nuclear proteins using 1 M urea, 1% NP-40 and 300 mM NaCl (4). Referred to as NUN (NaCl, urea, NP-40), this method also requires dialysis of the nuclear extract to remove the urea and the detergent, but it still results in a large percentage of denatured and inactive proteins in the extract.

Here, we compare these two methods to a third method we developed in our laboratory for the preparation of nuclear extracts. We have modified and streamlined a method for isolating nuclear proteins from rat liver in which the nuclei are isolated and lysed, followed by extraction of soluble nuclear

proteins with 0.4 M ammonium sulfate (AS) (3). The soluble protein fraction is then spin-dialyzed into a buffer compatible with EMSA analysis. This AS method for extracting nuclear proteins takes only about 90 minutes, allows for high sample throughput and generates extracts with high concentrations of intact nuclear proteins that are very active in EMSA analysis. The addition of the spin-dialysis step allows for the rapid removal of solutes that may interfere with EMSA, as well as for flexibility in the final buffer composition. We have found that not only is this method rapid and efficient in extracting DNA binding proteins, but it also extracts larger quantities of active proteins able to bind DNA in gel shift assays.

MATERIALS AND METHODS

Cell Lines

Mouse Ltk- aprt- cells were grown in DMEM (Life Technologies, Rockville, MD, USA) supplemented with

Short Technical Reports

10% calf serum (HyClone, Logan, UT, USA). S6 is a clone of Ltk- cells and can be induced by estradiol to express the transcription factor C/EBP α (K. D'Arigo and D.T. Kurtz, unpublished results).

Preparation of Nuclear Extracts from Cultured Cells

Nuclear extracts were prepared from approximately 2×10^7 cells/sample, though the protocol is successful when

used with as few as 1×10^6 cells. Cells were processed as shown in Table 1. For comparison, extracts were also prepared from nuclear pellets obtained as in Table 1, step 4, using either the modified Dignam method (1) or the NUN extraction method (4) and then spin-dialyzed as described in Table 1, step 6. Typically, from 2×10^7 cells, the AS method yields between 200–300 μ g protein, the NUN method yields 250–350 μ g and the modified Dignam method yields 100–150 μ g.

Preparation of Nuclear Extracts from Tissue Samples

As little as 0.5 g tissue was homogenized using a Dounce homogenizer in 1 mL polyamine buffer (Table 1) without added NP-40. A 10% solution of NP-40 was then added to a final concentration of 0.1% NP-40. Homogenate was placed on ice for 10–15 min, and the remainder of the protocol was followed as in Table 1.

EMSA

The following oligonucleotides were used for EMSA analysis: (i) an oligonucleotide corresponding to a C/EBP binding site in the $\alpha 2$ U-globulin promoter (5'-TGTTTTCGGAAT-GTAATG-3') (6); (ii) the PPRE site from the *Xenopus* acyl CoA oxidase promoter (5'-GGGACCAGGACAAA-GGTCACG-3'); (iii) a consensus SP1 binding site (5'-TCGGGGCGGGGC-GAGC-3'); or (iv) a TRE AP1 site (5'-CTTGATGACTCAGCCGGAA-3'). Nuclear proteins were incubated with a [32 P]-labeled oligonucleotide in 25 mM HEPES (pH 7.9), 100 mM KCl, 4% ficoll, 5 μ M ZnCl $_2$, 0.1 mM DTT, 0.05% NP-40, 5 mM MgCl $_2$, 1 μ g/mL BSA and 50 ng/ μ L poly dI-dC at 4°C (5). Nonspecific binding was assessed in the presence of a 100-fold excess of unlabeled oligonucleotide corresponding to the labeled oligonucleotide. The samples were subjected to electro-

Figure 1. EMSA analysis of nuclear extracts from mouse Ltk- cells prepared by three methods. Nuclear extracts from mouse Ltk- cells were prepared in parallel, and 2.5 μ g protein were used in EMSA analysis. Free probe (F) is indicated by the arrow at the left. (A) Binding to consensus C/EBP (lanes 1–7) or AP1 (lanes 8–14) sites. Lanes 1 and 8 contain free probe. Ltk- cell extracts were prepared by the AS method (lanes 2, 3, 9 and 10), a urea extraction method (NUN) (lanes 4, 5, 11 and 12) or the modified Dignam method (NaCl) (lanes 6, 7, 13 and 14). Nonspecific binding in the presence of unlabeled oligonucleotide is shown in alternate lanes. (B) Binding to consensus PPRE (lanes 1–7) or SP1 (lanes 8–13) site was examined. Lane 1 contains free probe. Ltk- cell extracts were prepared by NaCl extraction (lanes 2, 3, 8 and 9), NUN extraction (lanes 4, 5, 10 and 11) or AS extraction (lanes 6, 7, 12 and 13). Nonspecific binding in the presence of unlabeled oligonucleotide is shown in alternate lanes.

phoresis on 5% polyacrylamide gels at 4°C and visualized either by phosphoimaging (Molecular Dynamics, Sunnyvale, CA, USA) or autoradiography.

RESULTS AND DISCUSSION

Comparison of Nuclear Extract Preparation Methods

Nuclear extracts were prepared in parallel from mouse Ltk- cells using either the AS method, a urea extraction (NUN) or the modified Dignam high-salt method (NaCl). Equal protein amounts of extracts were subjected to EMSA with a variety of labeled oligonucleotide probes as shown in Figure 1. Increased binding of proteins to all probes was seen to varying degrees using extracts made by the AS method as compared with the NaCl or NUN methods. Increased binding of

DNA binding proteins to other probes has also been observed from AS extracts (data not shown).

The best example of the difference in the proteins extracted by the three different methods compared here is shown in Figure 1A. Mouse Ltk- cell extracts made by the NaCl or NUN method exhibit little or no binding to a consensus C/EBP oligonucleotide when 2–5 µg protein are subjected to EMSA analysis. However, these cells do produce the transcription factor C/EBPβ as detected by Western analysis of whole cell extracts (data not shown). When Ltk- nuclear extracts are prepared using the AS method, EMSA analysis of 2–5 µg protein results in a strong doublet of binding activity that corresponds to the two variant forms of C/EBPβ expressed in these cells, as can be shown by supershift analysis (data not shown). The use of either the NaCl or NUN method results in extracts con-

taining little or no binding of C/EBPβ to its consensus sequence. Similarly, EMSA analysis using consensus AP1, PPRE and SP1 oligonucleotides and equal amounts of protein from the different nuclear extracts shows that a higher percentage of active DNA binding proteins is present in nuclear extracts prepared by the AS method.

Practical Consequence of Improved Protein Yield

We have created a mouse fibroblast cell line, S6, capable of selective induction of C/EBPα using an estradiol-regulated expression system. In the absence of estradiol, cells express only the endogenous C/EBPβ seen in the parental mouse Ltk- cell line. However, after the addition of estradiol, cells also express the related transcription factor C/EBPα.

Nuclear extracts were prepared in

Short Technical Reports

Figure 2. EMSA analysis of nuclear extracts prepared from estradiol-induced SP6 cells by three methods. Nuclear extracts were made in parallel by different methods from S6 cells following treatment with estradiol and induction of the transcription factor C/EBP α . Protein (2.5 μ g) was used in EMSA analysis. Lane 1 contains free probe (F), corresponding to a consensus C/EBP binding sequence and is indicated by the arrow at the left. Extracts made from cells treated with estradiol (E₂) to induce C/EBP α expression are indicated by (+). Lanes 2–5 contain NaCl extracts; lanes 6–10 contain NUN extracts; and lanes 10–13 contain AS extracts. Nonspecific binding is assessed in the presence of unlabeled oligonucleotide in alternate lanes. Endogenous C/EBP β is shown by the double arrow at the left, and induced and slower migrating C/EBP α is indicated by the single arrow at the left.

parallel from induced and uninduced S6 cells and equal amounts of protein were analyzed by EMSA for binding to a consensus C/EBP binding site recognized by both C/EBP α and C/EBP β . As shown in Figure 2, C/EBP α is extracted to varying degrees by all three methods, with the NUN and AS sulfate methods yielding the greatest amount of C/EBP α binding activity. However, C/EBP β was only present in extracts produced by the AS method. When lane 10 is compared to lane 12 in Figure 2, a clear difference between the pattern of binding to the consensus C/EBP site is evident between the induced and uninduced S6 cell extracts, and it becomes evident that the major C/EBP isoform present in induced S6 cells is C/EBP α . This observation could not have been made had the extracts been prepared by either of the other two methods because the endoge-

nous C/EBP β binding activity is not extracted by NUN or NaCl methods.

Using the AS method to prepare nuclear extracts generates larger quantities of protein for EMSA analysis. However, in addition to quantity, extracts produced by this method appear to be enriched in DNA binding proteins that are still active and capable of recognizing cognate binding sequences. The use of a spin-dialysis step prevents the large increase in volume that can occur with standard dialysis methods and significantly shortens the procedure.

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