Two-step cross-linking method for identification of NF-κB gene network by chromatin immunoprecipitation

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The chromatin immunoprecipitation (ChIP) assay has recently been exploited as a powerful and versatile technique for probing protein-DNA interactions within the chromatin environment. In this method, intact cells are fixed with a reversible DNA-protein cross-linking agent (formaldehyde), and associated DNA is enriched by immunoprecipitating a target DNA binding protein. The bound DNA in the immune complexes is then used to identify that specific DNA binding protein's endogenous genomic targets. Nuclear factor κB (NF-κB) is a highly inducible transcription factor that controls genetic networks important for pathogen- or cytokine-induced inflammation, immune response, and cellular survival. In our studies of the genetic network under control of the inducible NF-κB transcription factor, we found that the conventional ChIP technique using a single formaldehyde cross-linking step did not reproducibly cross-link it to DNA. As a result, we have developed a novel ChIP assay using a two-step cross-linking procedure, incorporating N-hydroxysuccinimide (NHS)-ester-mediated protein-protein cross-linking prior to conventional DNA-protein cross-linking. We demonstrate that this technique is highly efficient, cross-linking virtually all NF-κB/Rel A into covalent complexes, resulting in quantitative and robust identification of inducible NF-κB family binding to a variety of validated NF-κB-dependent genomic targets. To demonstrate the general utility of this two-step cross-linking procedure, we performed enhanced capture of cytokine-inducible signal transducer and activator of transcription-3 (STAT3) binding to one of its known target genes. Our method represents a significant improvement in the efficiency of ChIP analysis in the study of endogenous targets for rare transcription factors.

INTRODUCTION

Coordinate changes in gene expression are temporally and spatially controlled to produce a variety of phenotypic changes important in normal development and disease (1). In eukaryotic cells, genetic elements are maintained in dynamic chromatin structures. The first order of chromatin structure involves DNA compacted by wrapping around core nucleosomes, which in turn are organized into higher order domains through the action of H1 linker histones, architectural proteins, acetylases, and other scaffolding proteins (1). Recently, it has been appreciated that this chromatin structure exerts significant effects over coordinated gene expression by controlling promoter access by inducible sequence-specific DNA binding proteins (2,3). Of relevance, many hormonal or developmentally controlled stimuli activate signal transduction networks controlling families of low abundance, sequence-specific DNA binding proteins (4–6). Because of their influence on cellular phenotype, the binding and targets of inducible transcription factors have been intensely investigated using a variety of biochemical and genomic approaches.

The techniques that have been classically used to this end are electromobility shift assays (EMSAs) and DNase I footprinting assays. The EMSA involves binding a radiolabeled oligonucleotide to either a stimulated nuclear protein extract or a recombinant transcription factor and resolving the resulting complexes by native polyacrylamide gel electrophoresis (PAGE) (7). The problems now recognized with this approach are that (*i*) the DNA-protein complex does not have the complexity that is seen in cellulo due to the lack of relevant chromatin structure; (*ii*) the DNA is often of insufficient length to measure complex interactions (e.g., DNA bending or looping); and (*iii*) DNA binding sites identified by EMSA poorly predict the presence of actual binding sites in cellulo (8). DNase I footprinting is used to identify the region of DNA bound by a transcription factor by assessing nucleotides resistant to nuclease attack. Although this technique can be adapted to understanding protein binding within its native chromatin context (9,10), the method is not sensitive to weak or partial DNA binding, and the precise identity of the protecting complex cannot be elucidated.

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The finding that the access of some sequence-specific transcription factors is tightly controlled by the chromatin environment of the target gene has stimulated development of other approaches to the analysis of protein-DNA interactions (2,3). Avoiding some of the shortfalls of EMSA and DNase I footprinting, the chromatin immunoprecipitation (ChIP) assay has been used to assay the binding of architectural DNA binding proteins, transcription factors, and members of the polymerase complex within native chromatin contexts (11,12). In this technique, intact cells are treated with formaldehyde (FA) to covalently link protein to DNA, the nucleoprotein complexes are then mechanically sheared and the cross-linked DNA-protein complexes enriched by immunoprecipitation. The retrieved complexes are then analyzed by PCR amplification to detect and quantify specific DNA targets.

The ChIP assay has been adopted as a powerful method for the analysis of proteins interacting within a native chromatin environment and is versatile enough for adaptation for a variety of purposes (13). This assay has been utilized in yeast (14), *drosophila* (15), tetrahymena (16), various mammalian cell lines, and even on whole mouse embryos (17,18) for the analysis of low abundance transcription factor binding. In addition to focused study of a single or group of genes, some have used this methodology for systematic promoter cloning (19,20) or identification of gene targets using promoter microarrays (14,21). ChIP has been used to determine the allele-specific transcription factor binding patterns (22) or measure long-range enhancer binding (23). One group has combined the ChIP technique with DNA footprinting methods (24), while another has devised ways of using ChIP for the analysis of RNA-protein interaction (25).

We sought to apply the ChIP methodology for network analysis of genes under control of the nuclear factor κB (NF-κB) transcription factor to study the precise timing of its binding in response to activating stimuli in a native chromatin context (6). NF-κB is a family of highly tumor necrosis factor (TNF)-inducible cytoplasmic transcription factors that controls genetic networks important in the hepatic acute phase response (26), immune response (27), atherosclerosis (28), and cellular survival pathways (29). Upon cytokine stimulation, the prototypical NF-κB complex, composed of 65 kDa Rel A • 50 kDa NF-κB1 heterodimers, enters the nucleus and binds to target gene promoters containing specific DNA binding sites. Although the mechanisms for NF-κB activation have been intensively investigated, relatively little is known about the global genomic targets of this transcription factor. Earlier we exploited a high-throughput analysis of expressing a tightly tetracycline-regulated dominant negative inhibitor to identify gene networks directly under NF-κB control (5,6,30). In spite of the dependence on NF-κB translocation for

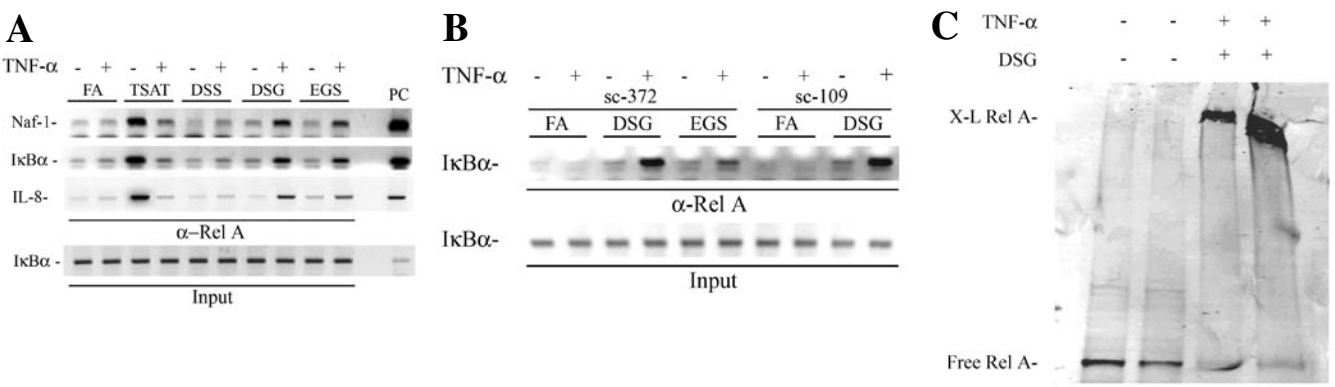

Figure 1. Efficiency of sequential protein and DNA cross-linking on inducible NF-κB/Rel A binding to endogenous promoters. (A) Effects of different cross-linking agents on NF-κB/Rel immunoprecipitation efficiency. A ChIP assay was performed by sequential protein-DNA cross-linking by various NHS-ester-containing cross-linking reagents (see Table 1) and compared with the immunoprecipitation efficiency of fixation by FA alone. Immunoprecipitations were performed on control (-) or TNF-stimulated (+) cells using anti-NF-κB/Rel A antibody. Enrichment in the immunoprecipitates for the Naf-1 (top panel), IκBα (middle panel), and IL-8 (lower panel) was detected by PCR. Samples were fractionated by agarose gel electrophoresis and detected by ethidium bromide staining. In FA cross-linked extracts, a 1.8-fold increase in the relative amount of Naf-1 promoter was seen after TNF treatment. The addition of DSG increased the relative amount of Naf-1 promoter recovery to 8.1-fold. Similarly, 5.5-fold and 7.1-fold increases in IκBα and IL-8 promoter sequences, respectively, were produced after TNF-α stimulation when compared with sequences recovered using FA alone. Input chromatin (10%) was decross-linked and assayed by PCR, indicating equivalent amounts were used in the immunoprecipitation (bottom panel). The experiment was performed in duplicate for the IκBα, IL-8, and Naf-1 targets. (B) Efficiency of N- or C-terminal-directed NF-κB/Rel A antibodies. N-terminal (sc-109) and C-terminal (sc-372) antibodies against NF-κB /Rel A were used in ChIP using FA or sequential protein-DNA cross-linking. Immunoprecipitated DNA was assayed for the enrichment of IκBα promoter binding by PCR (top panel). Input chromatin (10%) was decross-linked and assayed by PCR (bottom panel). For sc-372 antibody, a 1.2-fold TNF-induced increase IκBα promoter recovery was observed with FA, whereas a 2.6-fold increase was observed with DSG. A similar difference was observed for the sc-109 antibody. The experiment was done in duplicate. (C) DSG efficiently cross-links NF-κB/Rel A protein. Cells were treated with TNF-α for 0 (-) or 60 (+) min, fixed in the absence (-) or presence (+) of 2 mM DSG, and lysed in whole cell lysis buffer. Shown is a Western blot using 100 µg of protein fractionated on a continuous 8% denaturing PAGE gel, transferred to a PVDF membrane, and blotted with anti-NF-κB/Rel A (sc-372, 1:1000 dilution). X-L Rel A indicates cross-linked NF-κB/Rel A. Note the depletion of the uncross-linked (free) 65 kDa NF-κB/Rel A and its appearance as a smear of larger immunoreactive material at the top of the gel. The Western blot analysis was performed in quadruplicate; shown is a representative blot. NF-κB, nuclear factor κB; ChIP, chromatin immunoprecipitation; NHS, N-hydroxysuccinimide; TNF, tumor necrosis factor; IL-8, interleukin 8; FA, formaldehyde; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TSAT, Tris-succinimidyl aminotriacetate; DSS, disuccinimidyl suberate; DSG, disuccinimidyl glutarate; EGS, ethylene glycol bis(succinimidylsuccinate); PC, 25 ng genomic DNA positive PCR control.

expression of interleukin 8 (IL-8) (31), IκBα (32), CXCL-20/Exodus-1 (33), TNFAIP-3/Naf-1 (6), and NF-κB2 (34), and the presence of high-affinity binding sites in the promoters, we were surprisingly unable to identify NF-κB bound to these targets using conventional ChIP assay.

This report describes our successful development of a robust, quantitative ChIP technique using a novel two-step fixation technique. Specifically, we utilized the N-hydroxysuccinimide (NHS) ester chemistry of disuccinimidyl glutarate (DSG) to cross-link NF-κB to protein complexes before FA treatment to cross-link them to DNA. This technique is highly efficient, quantitative, and specific for the identification of inducible NF-κB family binding to bone fide NF-κB-dependent genomic targets. We provide evidence of this ChIP method's general applicability by enhanced detection of inducible STAT3 genomic targets.

MATERIALS AND METHODS

Cell Culture and Reagents

HeLa cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin G sodium, and 100 µg/mL streptomycin sulfate in a humidified atmosphere of 5% CO_2 as previously described (6). Homobifunctional NHS-ester cross-linking reagents, ethylene glycol bis(succinimidylsuccinate) (EGS), DSG, disuccinimidyl suberate (DSS), and Tris-succinimidyl aminotriacetate (TSAT) were purchased from Pierce Biotechnology (Rockford, IL, USA). Chemical properties of cross-linking reagents are shown in Table 1. Human recombinant TNF-α was obtained from Peprotech (Rocky Hill, NJ, USA). Cross-linking reagents were dissolved in 100% dimethyl sulfoxide (DMSO) at 0.5 M stock concentrations immediately prior to the addition, and unused reagent was discarded after use. FA, herring sperm DNA, DMSO, and bovine serum albumin (BSA) (fraction V) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Protein A-conjugated magnetic beads were from Dynal (Oslo, Norway). Rabbit polyclonal NF-κB/Rel A (SC-372, Sc-109), Rel B (SC-226), c-Rel (SC-70), and STAT3 (K15) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal NF-κB p50 and p52 were from Upstate (Charlottesville, VA, USA).

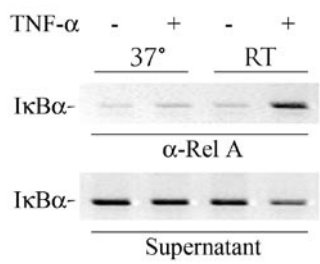

Figure 2. Temperature dependence of the formaldehyde fixation on ChIP performance. Cells were treated with TNF-α for either 0 (-) or 60 (+) min and cross-linked with DSG. The subsequent DNA cross-linking step in FA was conducted at either 37°C or at room temperature (RT; 22°C). Top panel, PCR for IκBα promoter enrichment after immunoprecipitation with NF-κB/Rel A (α-Rel A, sc-372). Bottom panel, chromatin in supernatant was decross-linked and assayed by PCR as a loading control. Note that fixation at room temperature was so efficient that depletion of IκBα was detected in the supernatant. The experiment was performed in duplicate with comparable results. ChIP, chromatin immunoprecipitation; TNF, tumor necrosis factor; DSG, disuccinimidyl glutarate; FA, formaldehyde; NF-κB, nuclear factor κB.

Two-Step Protein and Chromatin Fixation

One 100-mm dish per time point was seeded with 4–6 × 10^6 HeLa cells. After reattachment, the cells were serum deprived overnight in DMEM supplemented with 0.5% BSA. On the day of the experiment, the cells were stimulated with 30 ng/mL TNF-α for the indicated times so that all cells were harvested simultaneously. Cells were washed 3 times with room temperature PBS and 10 mL of PBS containing 1 mM $MgCl_2$, pH 8.0 (PBS/Mg) added to each plate. Magnesium-containing PBS was used to ensure that the monolayer remained attached during the fixation. NHS-ester stock solution was added to a final concentration of 2 mM and rapidly mixed. It is important to rapidly mix the NHS-ester because it has a tendency to form insoluble aggregates in aqueous solutions. Hela cells were fixed with DSG for 45 min at room temperature (22°C). The cells often became vacuolated during the course of fixation but remained adherent to the plate. At the end of fixation, the cells were washed 3 times with PBS. Ten milliliters of a freshly prepared solution of 1% (v/v) FA in PBS/Mg, pH 8.0, were added, and the cells were incubated at room temperature (22°C) for 15 min. Cells were rinsed 3 times with PBS, scraped, and transferred into 1.7 mL Eppendorf® tubes. Cells were lysed in 900 µL L1 buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.1% IGEPAL® 630 (Sigma-Aldrich), 10% glycerol, 1 mM dithiothreitol (DTT), supplemented with 1× mammalian protease inhibitor cocktail (PIC; Sigma-Aldrich) and 1 mM phenylmethysulfonyfluoride (PMSF) for 15 min on ice to remove free cytoplasmic proteins. Nuclei were precipitated by centrifugation (1200× *g* for 5 min) and resuspended in 500 µL sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) at room temperature. Chromatin was sonicated 4 times, at setting 4, for a 15-s duration each, with a 10-s break on ice between pulses on a Branson Sonifier 150 (Branson Ultrasonics, Danbury, CT, USA). The samples were centrifuged (5000× *g* for 5 min), and soluble chromatin was transferred to a fresh tube.

Average DNA length was verified using a 20-µL aliquot of soluble chromatin. FA cross-links were reversed by incubation at 65°C for 1 h in 200 mM NaCl, 0.5% SDS, 200 µg/mL proteinase K. The decross-linked DNA was then phenol-chloroform extracted and precipitated using ammonium acetate/ethanol. The DNA length averaged 300–400 bp after ethidium bromide staining of 1% agarose-Tris-borate-EDTA (TBE) gel.

Immunoprecipitation

Soluble chromatin was quantitated by measuring the absorbance at 260 nm, and equivalent amounts of input DNA were used in the immunoprecipitation, making sure that no more than 100 µL of the soluble chromatin

Table 1. Chemical Properties of Cross-Linking Agents Suitable for ChIP

Cross-Linker	Chemistry	Spacer Length	No. of Groups	Stock	Working (mM)
cisplatin	organometallic	4	2	100 mM	0.3–1.0
DMA	imidoester	8.6	2	N.A.[a]	10
DMP	imidoester	9.2	2	N.A.[a]	10
DMS	imidoester	11	2	N.A.[a]	10
DSG	NHS-ester	7.7	2	0.5 M	2
DSS	NHS-ester	11.4	2	0.5 M	2
EGS	NHS-ester	16.1	2	0.5 M	2
formaldehyde	methylene bridge	2	2	37%[b]	1%[b]
TSAT	NHS-ester	4.2	3	0.5 M	2

This is a tabulation of cross-linking agents either used for ChIP or that can potentially be used for ChIP in living cells. The cross-linking chemistry, spacer length, and suggested stock/working concentrations are listed. ChIP, chromatin immunoprecipitation; DMA, dimethyl adipimidate; DMP, dimethyl pimelimidate; DMS, dimethyl suberimidate; NHS, N-hydroxysuccinimide; TSAT, Tris-succinimidyl aminotriacetate; DSS, disuccinimidyl suberate; DSG, disuccinimidyl glutarate; EGS, ethylene glycol bis(succinimidylsuccinate).
[a]Not applicable because solubility issues prevent a concentrated stock.
[b]Thirty-seven percent by weight.

were added to each tube. Low ionic strength ChIP dilution buffer (50 mM NaCl, 10 mM HEPES, pH 7.4, 1% IGEPAL 630, 10% glycerol, 1 mM DTT, 1× PIC, and 1 mM PMSF) was added to a total volume of 900 µL. Four micrograms of antibody were then added and incubated overnight at 4°C with rotation. Magnetic protein A beads (Dynal) were washed and pre-equilibrated in low ionic strength ChIP dilution buffer in the presence of 100 µg/mL herring sperm DNA sonicated (3×, 15 s, at setting 2; Branson Sonifier 150) overnight at 4°C with rotation. Beads were washed twice with low ionic strength ChIP dilution buffer. Forty microliters of 50% bead slurry were added to each immunoprecipitation and incubated for 1–4 h at 4°C. In practice, this resulted in approximately a total of 12.5 A_{260} units of chromatin used as input for each immunoprecipitation. We have successfully used anywhere from 5–25 A_{260} units during the optimization of the technique and found that numbers near 12.5 gives the best sensitivity and specificity. It is critical, however, to ensure that the number of A_{260} units are kept as consistent as possible. Complexes attached to beads were captured using a magnetic stand (Dynal), rinsed twice with 500 µL low ionic strength ChIP dilution buffer, and transferred to a fresh tube. Beads were sequentially washed once with high-salt ChIP wash buffer (500 mM NaCl, 0.1% SDS, 1% IGEPAL 630, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0), once with LiCl ChIP wash buffer (0.25 M LiCl, 1% IGEPAL 630, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and twice with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Elution buffer (1% SDS, 0.1 M $NaHCO_3$) was freshly prepared, and precipitated complexes were eluted once with 250 µL for 15 min at 22°C, followed by a second elution with 50 µL (15 min at 22°C).

DNA Recovery and PCR Amplification

Eluates were combined and adjusted to 200 mM NaCl, 50 mM Tris-HCl, pH 6.8, 10 mM EDTA, 200 µg/mL proteinase K, and incubated at 65°C for 1–4 h. DNA was extracted by phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitated in the presence of ammonium acetate and 60 µg glycogen. Pellets were washed in 100% ethanol, dried, and resuspended in 30 µL of TE. Three microliters of DNA (10% total) were used as a starting point in PCR. Specific PCR primer sequences and reaction conditions can be found in Table 2.

RESULTS

Selection of Protein-Protein Cross-Linking Agents

In spite of the presence of functional, high-affinity binding sites in the Naf-1 and IκBα promoters (6,35,36), we were surprisingly unable to identify inducible NF-κB binding using conventional ChIP methods relying on FA-mediated cross-linking. Repeated attempts varying FA incubation times (from 15 min to 16 h), pH 7.0–9.0, and temperature (22°–37°C) of cross-linking yielded disappointing results (Figure 1A), even though these conditions produced robust cross-linking of modified histones to the IκBα promoter (data not shown). Moreover, although others have shown that DNA cross-linking using the organometallic cisplatin allowed the demonstration of ERp57 and actin binding to avian reticulocyte chromatin (37), this compound did not result in effective NF-κB cross-linking in our hands.

We were interested in a study where cross-linking using the bifunctional imidoester dimethyladipimate allowed detection of the yeast Rpd3

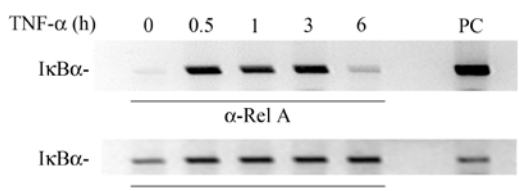

Figure 3. Time series for NF-κB/Rel A recruitment to the IκBα promoter. Cells stimulated with TNF-α for the indicated times (h) and cells fixed with sequential DSG-FA cross-linking. ChIP assay was then performed using anti-NF-κB/Rel A (sc-372, 4 µg). Shown is ethidium bromide stained agarose gel electrophoresis of the PCR products for the IκBα promoter (top panel). Input chromatin (10%) was decross-linked and assayed by PCR (bottom panel). PC, positive control, 25 ng genomic DNA. Relative to the control (0 h), a 3.7-fold increase in IκBα promoter abundance at 0.5 h was detected, with values at 1, 3, and 6 h being 3.6-, 4.2-, and 2.6-fold, respectively. The experiment was performed in triplicate with comparable results. A representative ethidium bromide stained agarose gel is shown. NF-κB, nuclear factor κB; TNF, tumor necrosis factor; DSG, disuccinimidyl glutarate; FA, formaldehyde; ChIP, chromatin immunoprecipitation.

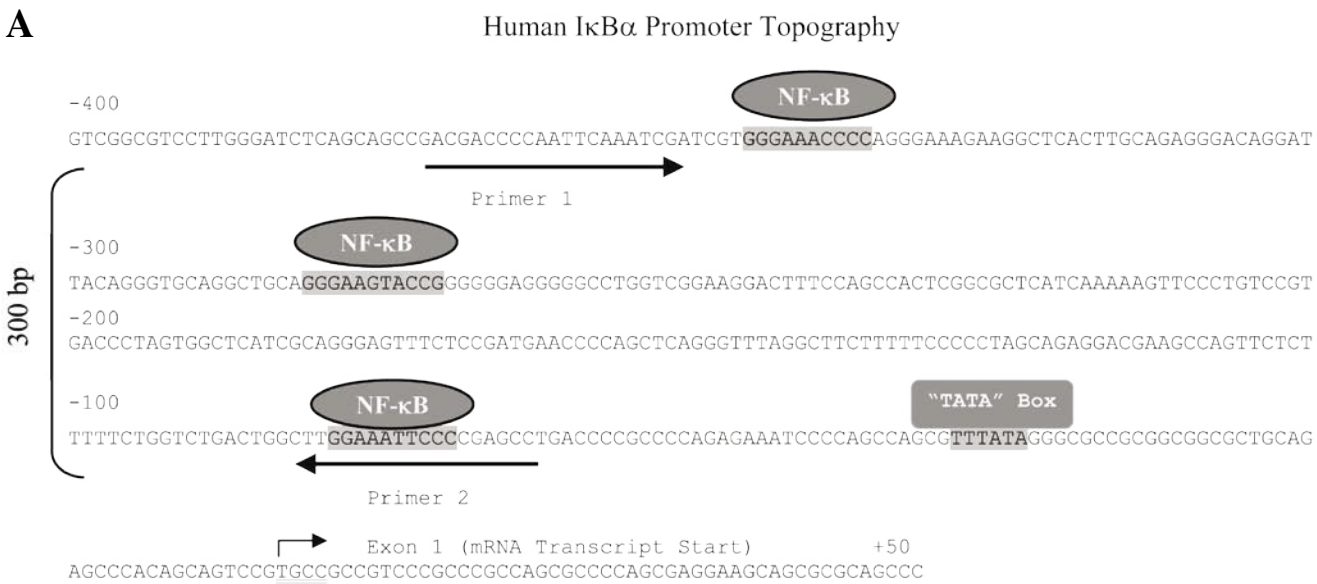

Figure 4. Detection of basal and TNF-induced NF-κB subunits binding the IκBα promoter. (A) Topology of IκBα promoter. Diagram of IκBα promoter with location of NF-κB binding sites. The bold arrows indicate the locations of primers used for ChIP PCR. Numbers refer to nucleotides upstream of the transcription start site. The double underline indicates the location of the messenger RNA (mRNA) transcription start site. (B) Effectivness of two-step protein-DNA cross-linking on NF-κB isoforms. Cells were stimulated with TNF-α for 0 (-) or 60 (+) min and sequentially cross-linked with DSG-FA. The ChIP assay was performed using 4 µg of the indicated NF-κB subunit-specific antibodies in immunoprecipitation. Enrichment for the IκBα promoter was detected by PCR. Nonspecific immunoglobulin G (IgG; top panel) served as a specificity control. Input chromatin (10%) was de-cross-linked and assayed by PCR (bottom panel). Negative control (NC) represents the PCR using no input DNA. Positive control (PC) was PCR using 25 ng genomic DNA. Fold increase in IκBα promoter recovery was 1.1, 8.9, 4.7, 3.4, and 2.1 for immunoprecipitation with IgG, Rel A, c-Rel, p50, and p52. Each experiment was performed at least in duplicate with representative ethidium bromide stained gels shown. TNF, tumor necrosis factor; NF-κB, nuclear factor κB; ChIP, chromatin immunoprecipitation; DSG, disuccinimidyl glutarate; FA, formaldehyde.

protein binding to target DNA by ChIP (14). Unfortunately, this, too, was ineffective for NF-κB (data not shown). We next surveyed other protein cross-linking agents that might improve recovery of NF-κB-bound DNA by immunoprecipitation. In contrast to the methylene bridge (FA) or imidoester [dimethyl adipimidate (DMA), dimethyl pimelimidate (DMP), and dimethyl suberimidate (DMS)] chemistries, the NHS-esters (DSG, DSS, EGS, and TSAT) were attractive to test. Homobifunctional NHS-ester compounds are readily cell-permeable, they have long half lives (4–5 h at pH 7.0) compared with their reaction time (45 min), they are reactive toward primary amines (making them effective protein-protein cross-linkers), and are readily commercially available (Table 1). The reaction chemistry is similar to imidoesters except that the generation of the soluble, free leaving group, N-hydroxysuccinimide, can be detected by absorbance at 260 nm. This ability to monitor the kinetics and extent of cross-linking is an additional advantage. Also, NHS-esters do not have the strict pH dependencies on cell permeability that are seen with imidoesters. We tested a series of NHS-esters using a two-step (protein-protein followed by protein-DNA) cross-linking with the notion that there may be an optimal distance between reactive primary amines that would increase NF-κB capture. Control or TNF-stimulated cells were subjected to ChIP using a single step (FA alone) versus a two-step cross-linking protocol. Although the DSS and TSAT precipitated in the aqueous PBS fixation solution in this experiment (making their effects indeterminate), we noted increased inducible enrichment of the Naf-1 promoter with DSS, DSG, and EGS (Figure 1A), with DSG producing optimal results. Moreover, enhanced NF-κB/Rel A binding was also detected on the IκBα and IL-8 promoters (Figure 1A, middle and lower panels), indicating that this effect was general for NF-κB targets.

Our next question was whether the two-step cross-linking procedure would result in epitope masking, thereby affecting the efficiency of the immuno-

precipitation. To test this idea, we performed the two-step ChIP technique using immunopreciptating antibodies specific to either the DNA binding (NH_2) or transactivating (COOH terminus) of NF-κB/Rel A. Chromatin prepared from cells fixed with FA alone, or the two-step cross-linking (EGS or DSG) was immunoprecipitated with NH_2 or COOH terminal NF-κB/Rel A antibodies (Figure 1B). For chromatin prepared by one-step FA cross-linking, no inducible NF-κB could be detected by either antibody; however, inducible enrichment for IκBα binding was again seen using DSG. These data suggest that the protein cross-linking does not result in significant masking of NF-κB/Rel A epitopes. To confirm the efficiency of DSG in cross-linking NF-κB/Rel A, the formation of the NF-κB/Rel A complexes was measured. For this, total cellular protein from control or TNF-stimulated cells exposed in the absence or presence of DSG was subjected to denaturing electrophoresis by SDS-PAGE fractionation, and NF-κB/Rel A complexes were detected by Western blot analysis (Figure 1C). The free 65 kDa NF-κB/Rel A band is completely up-shifted to larger protein complexes in the presence of DSG, independent of cellular stimulation. These observations indicate that DSG very efficiently cross-links NF-κB/Rel A under these conditions.

Temperature Effect on Formaldehyde Cross-Linking on Recovery of NF-κB/Rel A-Bound DNA

Because the kinetics and efficiency of FA-mediated cross-linking are temperature-dependent (13,38), we investigated the optimal temperature for cross-linking. Cells were subjected to identical DSG-mediated protein cross-linking and then fixed in FA at either room temperature (22°C) or 37°C for 15 min. Soluble chromatin was prepared, immunoprecipitated by anti-NF-κB/Rel A, and enrichment of NF-κB binding to the IκBα promoter was determined (Figure 2). These surprising findings show that recovery using room temperature fixation is more efficient than fixation at 37°C (Figure 2). During this experiment, we noted that the size of the insoluble chromatin pellet produced by the 37°C fixation condition was dramatically increased in size. We interpret this phenomenon to indicate that extensive FA fixation at 37°C produces NF-κB containing nucleoprotein complexes that remain insoluble after sonication. These complexes are removed by the subsequent centrifugation prior to the immunoprecipitation reaction, resulting in lower yields of target promoters in PCR.

The two-step cross-linking procedure was extremely efficient at cross-linking NF-κB to its target DNA. For example, we noted depletion of IκBα-containing DNA from the immunoprecipitation supernatant of the DSG-treated cells fixed at room temperature (Figure 2, bottom panel). These findings indicate the two-step cross-linking technique produces highly efficient cross-linking of NF-κB/Rel A to its target genes.

Performance of the Two-Step ChIP Method for Measurement of NF-κB/Rel A Binding Kinetics

Real-time PCR assays of TNF-α-stimulated cells generated in our laboratory indicate IκBα messenger RNA (mRNA) and transcription peak 1 h after stimulation, returning nearly to control levels by 6 h (6). Moreover, this induction is NF-κB dependent because the IκBα promoter is not induced by TNF when NF-κB/Rel A translocation is inhibited (6). To determine whether the assay would accurately measure the dynamics of NF-κB/Rel A binding, we applied the two-step ChIP assay to a time series of TNF-stimulated HeLa cells. Figure 3 shows binding of NF-κB/Rel A is strongly induced 30 min after TNF stimulation, persists for 3 h, and declines thereafter. This binding pattern closely follows the peak times of IκBα transcription and mRNA abundance, indicating that this

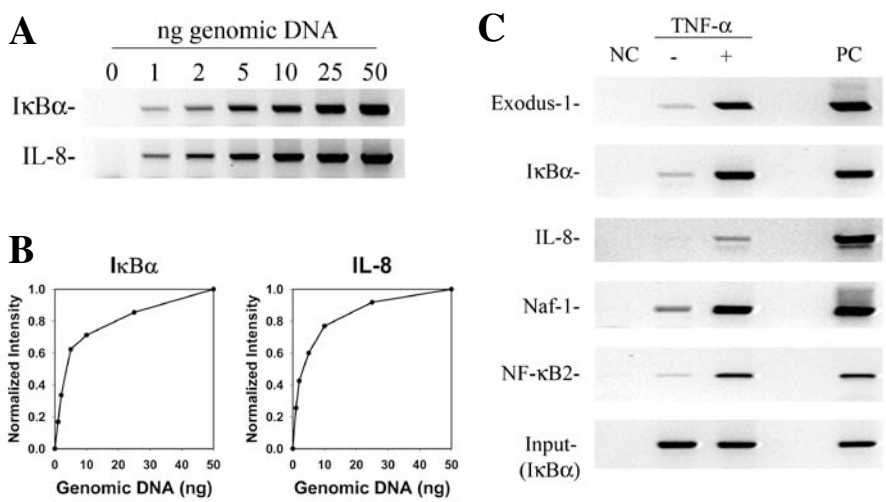

Figure 5. Sequential protein-DNA cross-linking ChIP method on detection of inducible NF-κB/Rel A binding to endogenous gene network. (A) PCR assay performance. Serial amounts (ranging from 0–50 ng) of genomic DNA were amplified by PCR using primers specific for human IκBα (top panel) and IL-8 promoters (bottom panels). Shown is an ethidium bromide stained agarose gel of the specific products. See Table 2 for the sequences of the primers used. Optimization PCR was performed exhaustively (at least 5 times) with representative gels shown. (B) Quantitation of PCR product as a function of input DNA. The absorbance of ethidium bromide stained PCR product was quantitated by phosphorimager analysis. Data are presented as a fraction of the maximal signal (produced by 50 ng genomic DNA) and plotted as a function of input genomic DNA. The quantitation reflects the gel shown in panel A. (C) TNF-inducible NF-κB/Rel A binding to endogenous target genes. Cells were stimulated with TNF-α for 0 (-) or 60 (+) min, sequentially cross-linked with DSG-FA, and chromatin immunoprecipitated with anti-NF-κB/Rel A (Rel A). Enrichment of Exodus-1, IκBα, IL-8, Naf-1, and NF-κB2 promoters was detected by PCR using promoter-specific primers (Table 2). Input chromatin was assayed using IκBα promoter-specific primers (bottom panel). NC, negative control; PC, 25 ng genomic DNA positive control. The TNF-induced fold increase in promoter recovery was 15.6-fold (Exodus-1), 9.3-fold (IκBα), 4.1-fold (IL-8), 3.5-fold (Naf-1), and 3.3-fold (NF-κB2). The experiment was performed in duplicate with comparable results. ChIP, chromatin immunoprecipitation; NF-κB, nuclear factor κB; IL-8, interleukin 8; TNF, tumor necrosis factor; DSG, disuccinimidyl glutarate; FA, formaldehyde.

Table 2. Primers, Genes, and PCR Conditions Used in the Study

Gene Name	GenBank® No.	Primers		Anneal		Extension		Product
		Sense	Antisense	Temp (°C)	Time (s)	Temp (°C)	Time (s)	(bp)
Exodus-1	U64197	5′-ACCCTGACCTTCGCACCTTC-3′	5′-CCGCAGAGGTGGAGTAGCAG-3′	64	30	68	75	313
IκBα	M69043	5′-GACGACCCCAATTCAAATCG-3′	5′-TCAGGCTCGGGGAATTTCC-3′	60	30	68	75	300
IL-8	M28130	5′-GTTGTAGTATGCCCCTAAGAG-3′	5′-GCCTTTGCATATATCAGACAG-3′	60	60	68	120	300
Naf-1	AJ011896	5′-GGTGTTTACCTCGCTCGGTC-3′	5′-GGGAGCTTGGGGACACAG-3′	64	30	68	75	280
NF-κB2	U20816	5′-CGTGAAAGACCCTCCTGTTC-3′	5′-AGAGCGAGATCCGGAGTTG-3′	64	30	68	75	285

Listed are the gene names, GenBank numbers, primer sequences, PCR conditions, and resulting product sizes used in the study. NF-κB, nuclear factor κB; IL-8, interleukin 8.

technique is suitable for analysis of the dynamics of NF-κB/Rel A binding to endogenous genes.

Two-Step ChIP Method Detects Inducible Binding of Other NF-κB Family Members

The NF-κB family includes the transcriptional activators NF-κB/Rel A and c-Rel, as well as the processed 50 kDa NF-κB1 (p50) and 52 kDa NF-κB2 (p52) (39). To explore the generality of the two-step technique, we examined whether it could specifically detect TNF-inducible binding of each of the NF-κB binding isoforms with the IκBα promoter (Figure 4). The location of the three major NF-κB binding sites in the IκBα promoter (relative to the PCR primers) are shown in Figure 4A. Figure 4B shows the results of two-step ChIP using antibodies for c-Rel, p50, and p52 subunits. In this experiment, immunoprecipitation specificity was determined using immunoglobulin G (IgG) as a control. Weak bands were produced in the IgG-stimulated extracts but, in all four cases, a TNF-induced enrichment for the IκBα promoter is readily apparent. These data indicate that this technique is generally applicable to the NF-κB subunits. Moreover, the assay is sensitive enough to detect the constitutive nuclear p50 and p52 (Figure 4) binding activity previously detected in unstimulated HeLa nuclear extracts by EMSA (6).

Detection of Multiple Endogenous NF-κB Genetic Targets

We next sought to determine whether the two-step ChIP assay could be used to detect multiple endogenous NF-κB-dependent targets. Our previous microarray studies using tetracycline-regulated cells identified CCL-20/Exodus-1, IκBα, IL-8,TNFAIP-3/Naf-1, and NF-κB2 as direct targets of NF-κB (6). For this, we first determined optimized conditions for semiquantitative PCR of these promoter fragments (Table 2). In Figure 5A, the performance of two representative PCR assays for IL-8 and IκBα are shown using various amounts from 0–50 ng of purified genomic DNA as template. In Figure 5B, product quantitation (from 5A) is plotted showing a dose-dependency for product occurring throughout the entire range of the experiment. We next used these semiquantitative PCRs to identify NF-κB/Rel A binding to the NF-κB genetic network (Figure 5C). In the TNF-treated samples, enrichment of the Exodus-1, IκBα, Naf-1, and NF-κB2 is so strong that the signal approximates that of 25 ng of genomic DNA used as a positive control. Again, these observations indicate that the two-step ChIP method is highly efficient at capturing NF-κB binding to diverse endogenous gene targets and is a quantitative and robust assay.

Two-Step ChIP Method Is Highly Efficient For Capture of STAT3 Target Genes

To establish its utility as a general method, we next determined whether the two-step ChIP method was efficient in capturing other inducible transcription factors. We have previously demonstrated that the interleukin 6 (IL-6) inducible transcription factor, STAT3, binds the target promoter, angiotensinogen (hAGT), in HepG2 hepatocellular carcinoma cells (40). HepG2 cells stimulated in the absence or presence of IL-6 were fixed with FA or DSG-FA and processed for ChIP using anti-STAT3 antibody. As shown in Figure 6, the two-step cross-linking procedure showed much stronger hAGT signal relative to that produced by one-step FA. Together, we conclude that the two-step cross-linking may be of general utility for inducible transcription factors.

DISCUSSION

The ChIP assay is a powerful, robust, and versatile method for the identification and measurement of DNA binding proteins to target genes within their native chromatin environment (13). However, this assay is empiric and is highly dependent on whether the fixation conditions efficiently trap the protein of interest to its target DNA sequence, which has limited our ability to measure NF-κB binding to target promoters. Our laboratory has made significant progress in this area by developing a two-step ChIP method that first stabilizes large multiprotein complexes with an NHS-ester-mediated protein-protein cross-linking followed by the conventional methylene bridge (FA)-mediated protein-DNA cross-linking reaction. Based on this study and our findings with STAT3, our method may be generally applied for the investigation of any low abundance transcription factor recognizing that the length of cross-link spacer may have to be empirically determined. In the example here, we observed a rank order of efficiency in NF-κB cross-linking of

DSG > EGS > DSS (Figure 1). Based on the length of its spacer, DSG forms cross-links over 7 Å whereas DSS spans 11.4 Å, and EGS, 16.4 Å. (The data regarding TSAT are indeterminate because of its insolubility in aqueous buffers.) Together, these findings indicate a cross-linking dependence on spacer lengths, which may vary depending on the target protein for which the assay is being developed. In this regard, it is fortunate that a vast variety of NHS-esters are available that span gaps of 4 to nearly 20 Å, making two-step cross-linking possible for almost any conceivable biologically relevant protein-protein interaction on DNA. It is important to note that NHS-esters have not been shown to directly react with DNA, probably due to the lack of accessible primary amine nitrogens in double-stranded DNA in cellulo.

Our data indicate that a largely unappreciated factor in the success of the two-step fixation ChIP assay is the temperature of FA fixation. Pilot studies in the efficiency of FA cross-linking in our laboratory indicated that cross-linking was dependent both on the temperature and time, with high temperatures and long time periods producing the most cross-linking. It has been apparent in our hands that there is a trade off between efficient DNA cross-linking and chromatin solubility. After high temperature fixation, the amount of insoluble chromatin appearing in the pellet after sonication is dramatically increased, even with high-intensity sonication. Therefore, it appears that much of the problem with high-temperature fixation stems from the difficulty in shearing such material into soluble fragments. In this case, relatively less sample survives the high-speed centrifugation to enter the immunoprecipitation reaction. The addition of more efficient mechanical fractionation, such as sonication with the addition of 425–600 µm glass beads, has been used by others (41,42), however, fortunately, we found those modifications unnecessary with room temperature fixation.

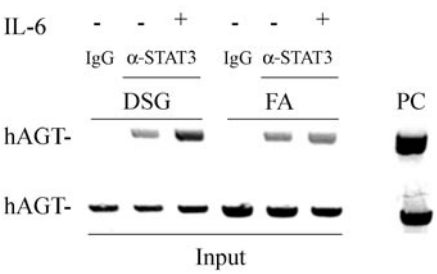

Figure 6. Comparison of ChIP cross-linking methods for STAT3 binding to the hAGT promoter. HepG2 cells starved overnight in 0.5% BSA and stimulated in the absence or presence of IL-6 (20 ng/mL) for 20 min. Cells prepared after FA, or sequential DSG-FA cross-linking and ChIP assay then performed using anti-STAT3 (N15, 4 µg). Shown is ethidium bromide stained agarose gel electrophoresis of the PCR products for the hAGT promoter (top panel). Input chromatin (10%) was decross-linked and assayed by PCR (bottom panel). PC, positive control, 25 ng genomic DNA. The experiment was performed in duplicate with similar results. ChIP, chromatin immunoprecipitation; STAT3, signal transducer and activator of transcription 3; BSA, bovine serum albumin; IL-6, interleukin 6; FA, formaldehyde; DSG, disuccinimidyl glutarate.

A final factor that must be addressed is the specificity and yield of the PCR analysis step. It is necessary to have a PCR that is very specific as well as very sensitive. In Figure 5A, we show PCRs that can detect to levels below 1 ng of genomic DNA with high specificity. In the case of the Naf-1 target, the detection threshold is in the tens to hundreds of picograms of genomic DNA (data not shown). PCR sensitivity in this range is necessary for the detection of the low amounts of DNA recovered when using the ChIP assay for rare transcription factors.

The one-step FA-mediated NF-κB ChIP assay has been shown by others (21,43–46) to be effective in a variety of cellular systems. It was perplexing to us why the numerous protocols presented in the literature could not be effectively reproduced in our hands. Of those papers surveyed using one-step cross-linking to detect NF-κB binding by ChIP, four basic protocols are used: (*i*) Upstate's Acetyl-Histone H4 Immunoprecipitation (ChIP) Assay Kit protocol (10 min FA fixation, followed by sonication and immunoprecipitation) (24,43,44,47); (*ii*) the CsCl gradient purification of complexes method involving fixation in excess of 45 min and a CsCl gradient purification step (18,21,41); (*iii*) highly variable empirical protocols involving times of fixation from 1 min to 2 h at temperatures from 0°–37°C and FA concentrations of 0.4%–1.5% (23,48,49); and (*iv*) any combination of the above three using nucleases rather than sonication to disrupt chromatin (50). We have made multiple attempts using conditions described from all three categories and have had uniformly disappointing results. In this regard, we have successfully cross-linked histones to DNA using FA-mediated cross-linking, indicating that the preparations of FA were biologically active. It is possible that cell-type-specific influences may have a significant effect on the efficiency of FA-mediated cross-linking because there is significant heterogeneity in the cell types used throughout the literature. However, we note that the DSG-FA ChIP has been applied in our laboratory to A549, HepG2, primary rat aortic and smooth muscle cells uniformly successfully with reproducible results and robust signal-to-noise ratios. Finally, there could be an interfering substance intrinsic to our reagents or systematic technical change that compromises our ability to reproduce the work of others.

We have shown that the two-step ChIP assay is sufficiently robust to show the dynamics of NF-κB/Rel A recruitment and dissociation from the IκBα promoter (Figure 3). This two-step ChIP assay allows us for the first time to confirm a mathematical prediction for the three distinct kinetic groups of NF-κB expression profiles that we have recently proposed (51). The assay is sufficiently sensitive and specific to detect the low levels of constitutive nuclear NF-κB p50 and p52 binding to the IκBα promoter in cellulo, a finding that we could previously only show as binding activity through EMSA (6). This further demonstrates the utility of this assay for the study of the kinetics of in cellulo NF-κB binding to target promoters.

In summary, we have developed a novel two-step ChIP assay for the analysis of NF-κB-dependent gene targets. Our findings presented here indicate that the efficiency of protein cross-linking is very high (Figure 1C), is free of detectable effects on epitope masking (Figure 1B), is useful for the detection of any NF-κB-bound promoter (Figure 5B), is suitable for analysis of NF-κB binding dynamics

(Figure 3), and at least four of the five NF-κB binding subunits can be quantitatively studied using this method, including NF-κB/Rel A, c-Rel, p50, and p52 (Figure 4).

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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