Video Article

Analysis of the c-KIT Ligand Promoter Using Chromatin Immunoprecipitation

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URL: https://www.jove.com/video/55689
DOI: doi:10.3791/55689

Keywords: Genetics, Issue 124, Chromatin immunoprecipitation, gene regulation, promoter, transcription factor, c-KIT ligand, TGF-β, SMAD

Date Published: 6/27/2017

Citation: Zhang, P., Rojas, A., Blechacz, B. Analysis of the c-KIT Ligand Promoter Using Chromatin Immunoprecipitation. *J. Vis. Exp.* (124), e55689, doi:10.3791/55689 (2017).

Abstract

Multiple cellular processes, including DNA replication and repair, DNA recombination, and gene expression, require interactions between proteins and DNA. Therefore, DNA-protein interactions regulate multiple physiological, pathophysiological, and biological functions, such as cell differentiation, cell proliferation, cell cycle control, chromosome stability, epigenetic gene regulation, and cell transformation. In eukaryotic cells, the DNA interacts with histone and nonhistone proteins and is condensed into chromatin. Several technical tools can be used to analyze DNA-protein interactions, such as the Electrophoresis (gel) Mobility Shift Assay (EMSA) and DNase I footprinting. However, these techniques analyze the protein-DNA interaction *in vitro*, not within the cellular context. Chromatin immunoprecipitation (ChIP) is a technique that captures proteins at their specific DNA binding sites, thereby allowing for the identification of DNA-protein interactions within their chromatin context. It is done by fixation of the DNA-protein interaction, followed by immunoprecipitation of the protein of interest. Subsequently, the genomic site that the protein was bound to is characterized. Here, we describe and discuss ChIP and demonstrate its analytical value for the identification of the Transforming Growth Factor-β (TGF-β)-induced binding of the transcription factor SMAD2 to SMAD Binding Elements (SBE) within the promoter region of the tyrosine-protein kinase Kit (c-KIT) receptor ligand Stem Cell Factor (SCF).

Video Link

The video component of this article can be found at https://www.jove.com/video/55689/

Introduction

In the nucleus of eukaryotes, DNA interacts with histone proteins and nonhistone proteins and is condensed into chromatin. In the physiological, pathophysiological, and cell biological context, cellular functions are spatially and temporarily controlled by chromatin-coordinated gene expression. DNA-protein interactions have an essential role in the regulation of cellular processes, such as DNA replication, recombination, and repair, as well as protein expression. Therefore, the analysis of DNA-protein interactions is an indispensable tool in the evaluation of gene expression and cell function.

Several techniques exist to assess DNA-protein interactions *in vitro*, such as the Electrophoresis (gel) Mobility Shift Assay (EMSA) and DNase I footprinting[1,2]. However, these techniques do not analyze the protein-DNA interaction within the chromatin and cellular context. ChIP is a technique that captures proteins bound to their specific DNA binding sites and thereby facilitates the identification of DNA-protein interactions within the chromatin context. The technique was originally developed by Gimour and Lis for the assessment of RNA polymerase II binding to specific genes in *Escherichia coli* and *Drosophila melanogaster*[3,4]. It is done by fixation of the DNA-protein complexes, followed by performing chromatin extraction and shearing the DNA into ~200 base pair (bp) fragments. Subsequently, the DNA-bound protein of interest is isolated by immunoprecipitation. After the reversal of the DNA-protein crosslink, the DNA is purified and analyzed. Several methods can be used for the analysis of the protein binding sites and depend upon the nucleic acid sequence of the protein binding site within the target gene[5]. In cases where the DNA sequence is known, standard Polymerase Chain Reactions (PCR) can be applied, using specific primer pairs flanking the known binding site. Quantitative Real-Time PCR (qRT-PCR) can also be used[6]. In cases where the sequence is unknown, ChIP can be combined with DNA microarrays (ChIP-on-chip), DNA sequencing (ChIP-seq), or cloning techniques[7,8,9].

The TGF-β pathway has potent tumor suppressing functions and is a key pathway in cell differentiation. It is activated through the binding of the TGF-β1 ligand to its cognate receptor complex, resulting in the serine-phosphorylation of SMAD2/3 transcription factors. Following their association with the common mediator, SMAD4, the SMAD-complex translocates to the nucleus and binds to the SBE within the promoter region of the target genes, where it regulates genes controlling the cell cycle, apoptosis, and cell differentiation. The transcriptional response to TGF-β stimulation is cell type- and context-specific[10]. Recently, we described a positive feedback loop between TGF-β and the c-KIT pathway[11]. In this model, TGF-β1-activated SMAD2 binds to the c-KIT ligand promoter and induces its expression and secretion. Subsequently, the c-KIT ligand activates the c-KIT receptor in an auto- and para-crinic fashion. c-KIT receptor activation results in STAT3 Tyr^{705}-phosphorylation via JAK1/2. Following STAT3-activation and nuclear translocation, STAT3 binds to the TGF-β1 ligand gene and regulates its expression.

Here, we demonstrate the essential role of ChIP analysis for the identification of SMAD2 binding to the c-KIT receptor ligand promoter and for the identification of the Signal Transducer (and) Activator (of) Transcription 3 (STAT3 binding to the TGF-β1-gene).

Protocol

1. Preparation of Solutions

1. **Prepare the following solutions fresh for each experiment.**
    1. For the **fixation solution**, prepare 37% formaldehyde and store it at RT. Dilute it in 1x Phosphate-Buffered Saline (PBS) to a final concentration of 1.42%.
    CAUTION: Formaldehyde is classified as irritant, corrosive, mutagenic, teratogenic, and carcinogenic. Do not ingest. Do not breathe gas/fumes/vapor/spray. In case of insufficient ventilation, wear suitable respiratory equipment. Avoid contact with skin and eyes. Keep away from incompatibles, such as oxidizing agents, reducing agents, acids, alkalis, and moisture.
    2. For the glycine stop-fix solution, prepare 0.125 M glycine in 1x PBS solution and store it at RT.
    3. For the cell scraping solution, prepare a 1x PBS solution in dH_2O and store it on ice. Directly before use, add proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 0.5 mM.
    CAUTION: PMSF is classified as corrosive and toxic; wear protective clothing and use it in a ventilated area.
    4. Directly before using the cell lysis buffer (20 mM Tris-HCl; pH 8.0, 85 mM KCl, and 0.5% NP40), add 1X Protease Inhibitor Cocktail (PIC; 100X stock solution in dimethyl sulfoxide (DMSO): 104 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 80 µM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin, and 1.5 mM pepstatin A) and 0.5 mM PMSF.
    5. Directly before using the nuclei lysis buffer (50 mM Tris-Cl; pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), and 1% SDS), add 1X PIC and 0.5 mM PMSF.

2. Cell Fixation and Shearing

1. Grow the cells to 70-80% confluence on 10 cm tissue culture plates. Stimulate the cells per the experimental aims.
2. Remove the tissue culture medium, add 5 mL of fixation solution, and incubate the cells for 10 min at RT on a shaking platform.
3. Remove the fixation solution and wash the cells twice with 10 mL of ice-cold 1x PBS.
4. Remove the 1x PBS, add 5 mL of glycine stop-fix solution, and incubate the cells for 5 min at RT on a shaking platform.
5. Remove the glycine stop-fix solution and wash the cells twice with 10 mL of ice-cold 1x PBS.
6. Remove the 1x PBS, add 2 mL of cell scraping solution, and harvest the cells using a cell scraper. Transfer the harvested cells to a conical tube on ice.
7. Centrifuge the samples for 10 min at 600 x g and 4 °C.
8. Remove the supernatant, resuspend the pellet in 1 mL of 1X cell lysis buffer, and incubate on ice for 30 min. Alternatively, snap-freeze the pellet in liquid nitrogen and store it at -80 °C.
9. Once the pellet is resuspended in the cell lysis buffer, transfer the cell suspension to an ice-cold dounce homogenizer and dounce for 10 strokes using a type-B pestle for cell disruption.
10. Transfer the cell suspension to a microcentrifuge tube and centrifuge the samples for 10 min at 2,400 x g and 4 °C.
11. Discard the supernatant, resuspend the pellet in 350 µL of nuclei digestion buffer, and incubate the samples for 5 min at 37 °C.
12. Add micrococcal nuclease (1 U/µL) and mix by vortexing. Incubate the samples at 37 °C for 5-20 min; mix the samples every 2 min by inversion.
    NOTE: The time and enzyme concentration differ between cell lines and might require optimization in cases of suboptimal enzymatic shearing.
13. **Alternatively, achieve chromatin shearing by sonication using commercially available sonicators.**
    NOTE: Sonication conditions will require optimization. An optimization example protocol is provided below using a sample volume of 300 µL and a sample processing power of 25%.
    1. Following step 2.10, discard the supernatant and resuspend the nuclear pellet in 1.0 mL of commercial shearing buffer.
    2. Aliquot 300 µL of the resuspended nuclear pellet into three 1.7 mL microcentrifuge tubes. Place them on ice.
    3. Shear the 3 aliquots of fixed chromatin at 25% power using 3 different conditions to determine the optimal sonication conditions:
    5 pulses of 20 s each, with a 30 s rest on ice between each pulse.
    10 pulses of 20 s each, with a 30 s rest on ice between each pulse.
    20 pulses of 20 s each, with a 30 s rest on ice between each pulse.
    4. Continue with Step 2.15, as outlined below.
14. Following the incubation with the micrococcal nuclease, add 7 µL of ice-cold 0.5 M EDTA to stop the reaction.
15. Centrifuge the samples with the sheared DNA for 10 min at 16,200 x g and 4 °C.
16. Transfer the sheared DNA-containing supernatant to a fresh microcentrifuge tube. Aliquot 50 µL into a separate microcentrifuge tube to confirm the successful shearing of the DNA (section 3). Use the remaining volume immediately for immunoprecipitation (section 4) or store it at -80 °C.

3. Confirmation of the Shearing Efficiency

1. Add 150 µL of nuclease-free dH_2O and 10 µL of 5 M NaCl to the 50 µL aliquot of the sheared chromatin from step 2.16.
2. Incubate the samples at 65 °C for 4 h to reverse the crosslinks.
3. Add 2 µL of RNase A (10 µg/µL) and incubate the samples at 37 °C for 15 min.
4. Add 10 µL of proteinase K (0.5 µg/µL) and incubate the samples at 42 °C for 90 min.

5. **For purification of the DNA, perform a phenol/chloroform extraction[12].**
    1. Add nuclease-free water to a final volume of 200 µL. Add 200 µL of phenol:chloroform:isoamyl alcohol (25:24:1) to the sample and vortex for 20 s.
    2. Centrifuge at room temperature for 5 min at 16,000 x g. Carefully remove the upper aqueous phase and transfer it to a fresh tube. Be sure not to carry over any phenol during the pipetting.
    3. Add 0.1 volumes of 3 M sodium acetate (NH_4OAc), pH 5.2, and mix by flicking the tube several times with a finger. Add 2.5 volumes of ice-cold 100% ethanol and mix by vortexing for 5 s. Incubate the sample O/N at -20 °C, or for at least 1 h at -80 °C.
    4. Centrifuge the sample for 30 min at 4 °C and 16,000 x g to pellet the cDNA.
    5. Carefully remove the supernatant without disrupting the pellet. Add 1 mL of RT, 70% ethanol and invert the tube several times.
    6. Centrifuge the sample for 2 min at 4 °C and 16,000 x g. Carefully remove the supernatant.
    7. Dry the pellet for 5-10 min at RT. Resuspend the pellet in 30 µL of dH_2O.
6. Use a 5 and 10 µL aliquot of the purified DNA for gel electrophoresis with a 1% TAE agarose gel.
   NOTE: Successful chromatin shearing results in a 200-1,500 bp DNA pattern. If the shearing is unsuccessful, determine the optimal shearing conditions for the specific cells by modifying the incubation time of the shearing step (step 2.12) to 5, 10, and 15 min.
7. Use the remaining sample to analyze the DNA concentration of the sample. Reverse-calculate the DNA concentration in the sheared chromatin sample (step 2.16); use the same DNA amounts for each treatment group for the immunoprecipitation (section 4).

4. Immunoprecipitation

1. Combine 10-25 µg of the sheared, crosslinked chromatin (step 2.16) with 25 µL of ChIP-grade protein G magnetic beads, 10 µL of immunoprecipitation dilution buffer (stock solution: 0.01% SDS, 1.1% Triton X-100, 16.7 mM Tris-HCl; pH 8.0, 1.2 mM EDTA, and 167 mM NaCl), and 1 µL of PIC.
2. After 4 h of incubation, add 1-10 µg of antibody (the antibody concentration will vary based upon the affinity of the antibody; it should initially be used per manufacturer recommendations and experimentally optimized if necessary) to a 1.5 mL microcentrifuge tube and add dH_2O to a final volume of 100 µL.
   NOTE: Instead of the above-described direct immunoprecipitation method, the indirect immunoprecipiation method can be used by first combining the chromatin with the antibodies and subsequently adding protein G magnetic beads.
3. Incubate the samples on an end-to-end rotator for 4-12 h at 4 °C.

5. Elution

1. Place the tubes on a magnetic stand. Once the magnetic beads aggregate on the side of the tube, carefully remove and discard the supernatant.
2. Wash the beads three times with 800 µL of wash buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8, 20 mM Tris-HCl; pH 8, and 150 mM NaCl); mix by inverting the tube several times. Place the tube on the magnetic stand and carefully remove and discard the wash buffer once the magnetic beads aggregate on the side of the tube.
3. Wash the beads once with 800 µL of wash buffer 2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA; pH 8, 20 mM Tris-HCl pH 8, and 500 mM NaCl); mix by inverting the tube several times. Place the tube on the magnetic stand and carefully remove and discard the wash buffer once the magnetic beads aggregate on the side of the tube.
4. Resuspend the beads in 50 µL of elution buffer (1% SDS and 100 mM $NaHCO_3$) and incubate the samples on an end-to-end rotator for 15 min at RT.
5. Place the tubes on a magnetic stand, and once the magnetic beads collect at the side of the tube, transfer the supernatant to a fresh tube.
6. Add 6 µL of 5 M NaCl, to reverse the crosslinking, and 2 µL of proteinase K (0.5 µg/µL). After mixing the samples, incubate the samples for 2 h at 65 °C.
7. For purification of the DNA, perform a phenol/chloroform extraction[12] (step 3.5) and subsequently resuspend the pellet in 30 µL of dH_2O.
   NOTE: The samples can be directly analyzed for protein binding sites (section 5) or can be stored at -20 °C.

6. Binding-site Analysis

1. Perform an analysis for specific binding sites within a known nucleic acid sequence using PCR or qRT-PCR. For primer design, follow conventional PCR or qRT-PCR protocols. Design the primers to synthesize a 150-400 bp amplicon that bridges the protein binding site of interest (**Figure 1**).
2. Set up the PCR using four different DNA templates to assure specificity: i) DNA from ChIP immunoprecipitated with the antibody of interest, ii) DNA from ChIP immunoprecipitated with an unspecific IgG antibody, iii) input DNA, and iv) H_2O as a negative control for the PCR to rule out contamination. In the case of pathway stimulation, choose a fifth template using a different set of primers specific to a known binding site of the immunoprecipitated protein.
   NOTE: In the case of the c-KIT ligand promoter analysis, use conventional PCR to demonstrate TGF-β-induced SMAD binding at the SBE within the c-KIT ligand promoter region. As a positive control, perform PCR on plasminogen activator inhibitor-1 (PAI-1) as a known target of TGF-β-induced SMAD binding.

Representative Results

The binding of the TGF-β1 ligand to its cognate receptor complex results in the serine-phosphorylation of SMAD2/3 transcription factors, followed by their association with the common mediator, SMAD4. The SMAD complex translocates to the nucleus. TGF-β can regulate gene transcription, either directly via SMAD binding to SBEs within regulatory regions of the target genes, or indirectly through the SMAD-regulated expression of transcriptional activators or repressors that subsequently regulate the expression of the gene of interest[10]. To test if the c-KIT ligand SCF is transcriptionally regulated by the direct TGF-β1-induced binding of SMAD2 to its promoter, we analyzed the SCF promoter-containing, 2.4-kb, 5'-flanking region of the SCF gene for the SMAD2 binding motif, 5'-AGAC-3' (SBE)[13,14]. We identified 7 putative SBEs upstream of the SCF start codon (**Figure 1A**). To confirm the TGF-β1-induced binding of SMAD2 to the SCF promoter, the ChIP assay described here was performed. The TGF-β1 treatment resulted in SMAD2 binding to the SCF promoter in human liver cancer lines, HepG2 and Hep3B, cells (**Figure 1B**). In the absence of TGF-β1-stimulation, no SMAD binding was noted. As a positive control for TGF-β1-induced SMAD activation, ChIP for PAI-1 was performed. The PAI-1 promoter is a known target of TGF-β/SMAD[13]. To confirm the specificity of the SMAD2 immunoprecipitation, unspecific IgG antibodies were used; no SMAD2 binding to the SBE was noted after immunoprecipitation with IgG.

For another demonstration of the ChIP technology, we analyzed the 5'-flanking region of the TGF-β ligand gene for the STAT3 consensus binding motifs 5'-TT(N4)AA-3' and 5'-TT(N5)AA-3'[15]. We identified two putative STAT3 binding sites upstream of the TGFB start codon at positions -4384/-4373 (STB-1) and -5365/-5357 (STB-2) (**Figure 2A**). To confirm the TGF-β1-induced binding of STAT3 to the TGF-β ligand gene, we performed ChIP assays using TGF-β1-treated HepG2 and Hep3B cells. TGF-β1 treatment resulted in STAT3 binding to the second putative STAT3 binding site (STB-2) of the TGFB gene, but not to the first one (STB-1) (**Figure 2B**). In this example, the positive STAT3 binding to STB-2 serves as an internal positive control. Similar to the above ChIP experiment, STAT3 binding to the STB-2 was not seen after immunoprecipitation using IgG.

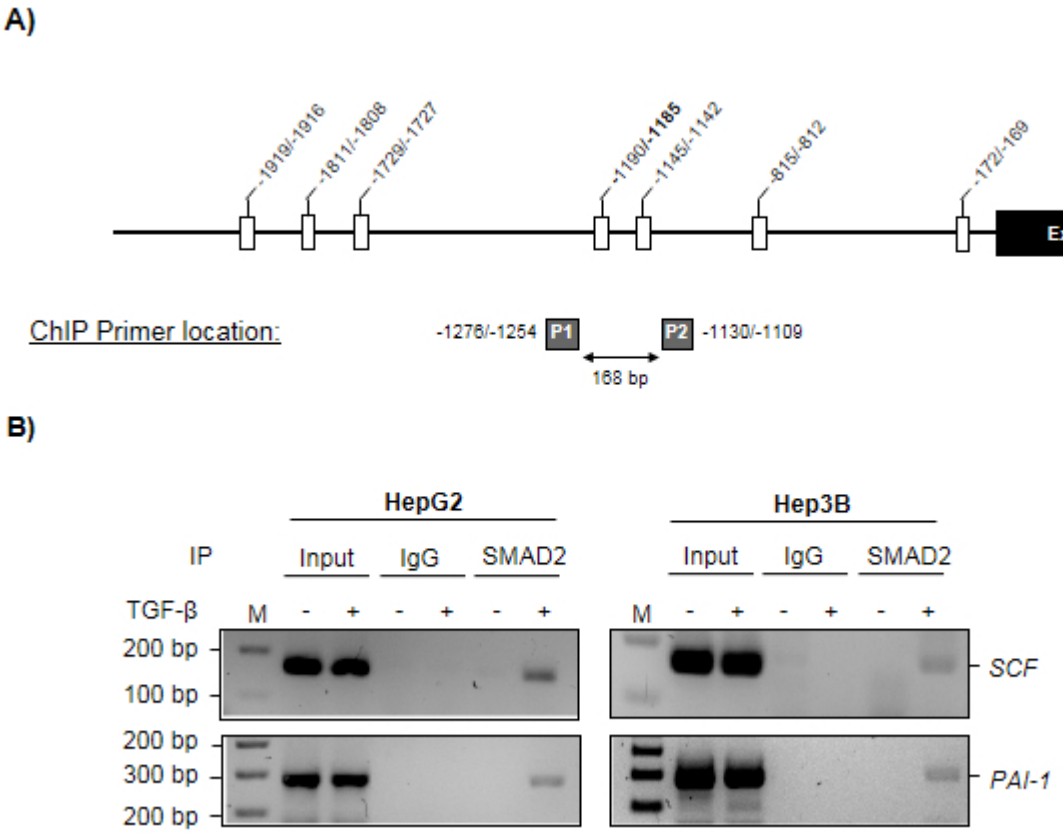

Figure 1: TGF-β-induced SMAD2 Binding to the SCF Promoter. (**A**) Schematic representation of the *SCF* promoter, with putative SBEs shown as boxes (white boxes = AGAC) with their relative position to the start codon. The ChIP primer location is shown below, with the relative positions to the *SCF* start codon and the PCR product size. (**B**) ChIP for SMAD2 binding to the *SCF* promoter. Chromatin-protein complexes of TGF-β1-treated and untreated HepG2 and Hep3B cells were immunoprecipitated with anti-SMAD2 antibody. PCR was performed using SCF-specific primers. On the left, PCR results using input DNA are shown. In the middle, PCR results after immunoprecipitation with unspecific IgG are shown for the confirmation of SMAD2 immunoprecipitation specificity. Shown in the bottom panel, ChIP for SMAD2 binding to *PAI-1* was used as a positive control. Please click here to view a larger version of this figure.

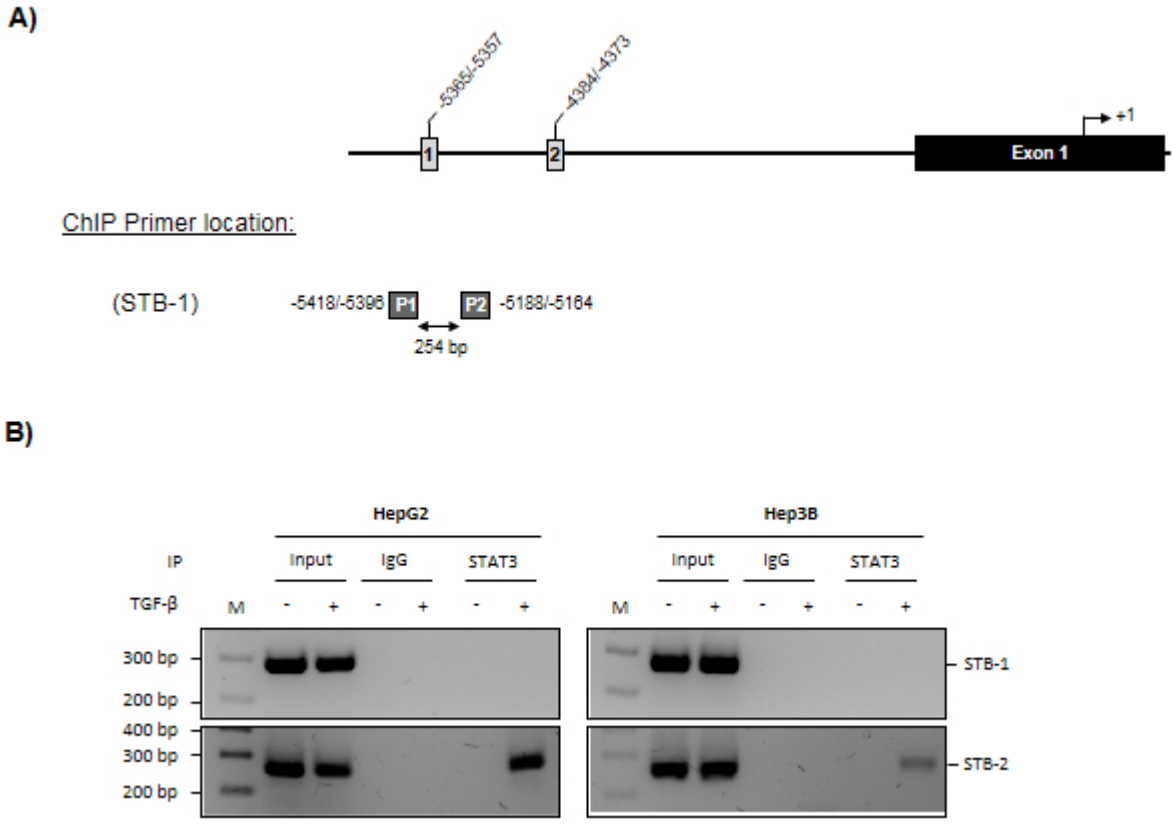

Figure 2: TGF-β-induced STAT3 Binding to the TGF-β Gene. (**A**) Schematic of the TGF-β gene, with putative STAT3 binding sites shown as gray boxes with their relative position to the start codon. ChIP primer locations are shown with their relative positions to the TGF-β start codon and the PCR product sizes indicated below. (**B**) ChIP for TGF-β1-induced STAT3 binding to the TGF-β gene. Chromatin-protein complexes of TGF-β1-treated and untreated HepG2 and Hep3B cells were immunoprecipitated with anti-STAT3 antibody. PCR was performed using TGF-β-specific primers. On the left, the PCR results using input DNA are shown. In the middle, the PCR results after immunoprecipitation with unspecific IgG are shown for confirmation of the STAT3 immunoprecipitation specificity. The upper panel shows the PCR results using primers specific for STAT3 binding site 1 (STB-1), and the lower panel shows the results for STAT3 binding to STAT3 binding site 2 (STB-2). Please click here to view a larger version of this figure.

Discussion

In this report, we demonstrate the TGF-β1-induced binding of SMAD2 to an SBE within the c-KIT ligand promoter and TGF-β1-induced binding of STAT3 to its recognition sequence within the TGF-β1 ligand gene. We demonstrate cytokine-induced binding of both transcription factors using chromatin immunoprecipitation.

Chromatin immunoprecipitation is a powerful tool to demonstrate the direct binding of a protein of interest to DNA, to characterize the stimuli that induce protein binding to DNA, and to characterize the DNA sequence to which the protein binds. The latter information can help in the identification of genes regulated by a specific protein of interest and is achieved by the use of ChIP-on-chip, ChIP-seq, or cloning strategies[7,8,9]. One of the major advantages of ChIP versus other methods of demonstrating DNA-protein binding, such as EMSA or DNase I footprinting, is that, in the ChIP technology, the binding is captured *in vivo*, while with the others, it is performed *in vitro*. Hence, ChIP provides insight into DNA-protein binding within the cellular context, while the other techniques represent an isolated system.

However, ChIP analysis is complex, involves multiple steps that can impact the results, and requires optimization and experience. One of the first steps that is critical for successful ChIP is the crosslink step (step 2.2). UV crosslinking is irreversible and therefore unsuitable for ChIP. Formaldehyde crosslinking is preferred, but formaldehyde concentration and crosslinking time can both influence the efficiency of chromatin shearing and antigen precipitation. In general, lower formaldehyde concentrations (1% w/v) and shorter crosslinking times (5-10 min) are preferable, as they improve the shearing efficiency. However, formaldehyde is not efficient at protein-protein crosslinking and therefore is suboptimal for proteins that do not directly bind to DNA[16]. For such cases, ChIP can be done in a two-step approach, in which protein-protein crosslinking is done first using crosslinkers such as disuccinimidyl glutarate, followed by formaldehyde-mediated DNA-protein crosslinking[17].

The next critical step is chromatin shearing. In our study, we used enzymatic shearing using micrococcal nuclease. Enzymatic shearing is especially useful for noncrosslinked native ChIP (N-ChIP), when sonication would disrupt the DNA-protein binding. N-ChIP is predominantly used for the analysis of histones and histone modifiers[18]. While micrococcal nuclease is considered a relatively non-specific endo-exonuclease, it has been shown to elicit sequence-specific cleavage[19]. This can result in a sequence-dependent bias in the resulting fragments, with

overrepresentation of certain gene loci[20]. Sonication creates randomly sized DNA fragments, without sequence bias, and is generally preferred for crosslinked ChIP (X-ChIP). However, sonication conditions must be determined empirically for each cell or tissue type and sonicator model, and resulting DNA fragments are generally larger than with enzymatic shearing[16]. Also, over-sonication or emulsification of the sample can result in the loss of antibody epitopes due to protein denaturation and degradation.

The immunoprecipitation step is another critical step that can greatly influence the results of ChIP. Agarose beads bind DNA nonspecifically, and variation in the number of added beads can affect the specific signal-to-background ratio. Hence, it is important to keep the agarose bead "slurry" well-suspended when it is added to the DNA-protein samples. In regard to the antibody used for the immunoprecipitation, in general, ChIP-grade antibodies should be used and, in cases where these are not available, at least immunoprecipitation-grade antibodies should be preferred. As specific epitopes can be masked during crosslinking, polyclonal antibodies are advantageous, as they recognize several epitopes. The amount of antibody added should be in excess of the factor being precipitated and thus should be determined empirically for each factor/antibody. Also, as the kinetics for reaching the equilibrium of antibody binding differ for each antibody, the optimal incubation conditions must be determined for each antibody.

Several controls can and should be included in the experimental setup. In cases where the induction of a specific DNA-protein binding reaction is evaluated, it is important to include an input DNA control to demonstrate equal template DNA amounts in the final analysis (*i.e.* PCR or qRT-PCR). An antibody control is required to confirm the specificity of the immunoprecipitation of the protein of interest. Usually isotype-matched immunoglobulins are well-suited as negative controls, but agarose beads can also be used. Occasionally, positive controls are used to confirm a functional experimental flow of the ChIP, and anti-histone antibodies are frequently used for this purpose. In stimulation experiments, a preferable positive control is immunoprecipitation of the protein of interest, with subsequent sequence analysis for a known DNA region the proteins bind to upon stimulation. In our representative results, the SBE within the PAI-1 promoter was used as a known target for TGF-β1-induced SMAD binding. In experiments without stimulated protein binding, a DNA sequence known not to be a target for the protein of interest can be used for the subsequent DNA analysis. In regard to the DNA analysis, it is important to include a reaction without template DNA to rule out contamination.

ChIP is an excellent technique to directly demonstrate the binding of a protein of interest to a gene. However, it is not a functional study. In cases in which a protein of interest is thought of having a regulatory role, it is imperative to also perform functional assays, such as reporter gene-based assays (*e.g.,* luciferase). In these protocols, the gene of interest is cloned in a regulatory position of a reporter gene. Induction of the gene of interest results in the expression of the reporter gene if it has a regulatory function. For further confirmation of the regulatory role of the specific protein of interest, either knock-down or knock-in cells can be generated in which the DNA-binding protein is genetically silenced. As an additional control, the protein binding site in the regulatory gene can be mutated to prevent the binding of the protein of interest. In either of the latter two experimental designs, cell stimulation will not result in the expression of the marker gene.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

This work was supported by the University of Texas MD Anderson Cancer Center, Houston, TX (startup funds, B.B.).

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