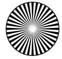
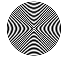

11 Chromatin Immunoprecipitation for *In Vivo* Studies of Transcriptional Regulation during Development

Daniel R. Buchholz, Bindu D. Paul, and Yun-Bo Shi

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I. INTRODUCTION

A. IMPORTANCE OF DETECTING PROTEIN/DNA INTERACTIONS IN THE CONTEXT OF CHROMATIN *IN VIVO*

Protein/DNA interactions form the molecular basis of transcriptional regulation in development and disease. Transcription is a complex process involving a large number of sequence-specific transcription factors and cofactors, and studies of the spatiotemporal nature of protein/DNA interactions and the consequences for chromatin remodeling are crucial for understanding the molecular mechanisms of transcriptional regulation *in vivo*. Previous techniques such as gel shift assays, though important for showing the capability of proteins to bind DNA *in vitro*, could not show whether these interactions actually take place in the context of chromatin in a cell or tissue, especially when considering tissue-specific gene regulation. The chromatin immunoprecipitation (ChIP) assay provides a snapshot of these critical protein/DNA interactions as they occur *in situ* (Kuo and Allis, 1999). The ChIP assay essentially involves formaldehyde crosslinking of proteins to each other and, importantly, to the site of DNA interaction. This crosslinking is followed by sonication to yield small fragments of chromatin, which are then immunoprecipitated using antibodies against protein components of interest to isolate pieces of DNA, bound to or associated with the protein. This DNA is then purified and analyzed by PCR-based methods.

ED: Change to "cross-linking" throughout?

B. MOLECULAR ANALYSIS OF GENE REGULATION, *IN VITRO* VS. *IN VIVO*

The ChIP assay was first developed in yeast and tissue culture cells and yielded many successes in understanding the role of transcription factors and cofactors in histone modifications and recruitment of basal transcription machinery (Kuo and Allis, 1999; Das et al., 2004). These studies provide a starting point for *in vivo* studies in molecular mechanisms of transcription during development because *in vitro* studies using cell lines, though derived from tissues, do not necessarily reflect normal cellular physiology, including gene regulation and transcription factor binding to promoters. Each cell type has its own set of transcribed genes and associated transcription factors, and even if the same gene is expressed in more than one cell type, there may be different molecular mechanisms regulating the gene given the presence of different or different levels of transcription factors and cofactors. More importantly, developmental or disease processes are not readily replicated in culture, making it necessary to analyze molecular mechanisms of gene regulation in animals *in vivo*.

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C. Brief Summary of the Use of Chromatin Immunoprecipitation *In Vivo*

The ChIP assay has become a standard procedure in cell culture, as reflected by published overviews and methods (Damjanovski et al., 2002; Farnham and Weinmann, 2002; Spencer et al., 2003), and protocols are widely available from companies such as Upstate Biotechnology, Inc. Using this assay to study protein/DNA interactions in tissues presents the added complications due to lack of single cell suspension and the fact that each tissue may have different requirements for fixing chromatin due to differences in composition of extracellular matrix and connective tissue. The protocols for tissues have been modified from protocols for cells (Parrizas et al., 2001; Wells and Farnham, 2002; Chaya and Zaret, 2004). These modifications have improved on methods for fixing chromatin with minimum disturbance of protein/DNA interactions while at the same time enabling isolation of fixed chromatin fragments from the tissue. These protocols for tissues converge with protocols for cells at the point of sonication after tissue nuclei are lysed with SDS.

D. Thyroid Hormone Receptor in Frog Metamorphosis as an Example

We have been using frog metamorphosis as a model to study the role of thyroid hormone (T3) receptors (TRs) in gene regulation and development (Sachs and Shi, 2000; Sachs et al., 2002; Buchholz et al., 2003; Buchholz et al., 2004). Frog metamorphosis is a postembryonic process controlled by T3, which exerts its effects on various target tissues via binding to TRs (Shi, 1999). The expression of TRs in premetamorphic tadpoles before, as well as after, the start of T3 secretion into the blood motivated development of a dual-function model for the role of TR in development (Sachs et al., 2000). TR expression during premetamorphosis is hypothesized to function by recruiting corepressors to repress T3-regulated genes, allowing the tadpole to grow. During natural metamorphosis or T3-induced metamorphosis, TRs function as activators to induce expression of T3-regulated genes necessary for the larval to juvenile transition by recruiting coactivators to the promoters. By using the ChIP protocol described below, we have been able to investigate different aspects of the model, such as the binding of TR to DNA, the recruitment of transcriptional cofactors, and changes in histone acetylation at T3 target genes in whole tadpoles or specific tissues during development. The methods described here should be applicable to other tissues and developmental systems, as well.

II. METHODOLOGICAL OVERVIEW

A. Study of Transcription Factors

Tissue-specific gene regulation largely depends on the set of transcription factors expressed in the cells and their binding to promoter regions in chromatin. The ChIP assay can directly assess these protein/DNA interactions in a promoter-specific, tissue-specific, and hormone- or developmental stage-dependent fashion (Figure

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11.1). We isolated fixed chromatin from intestines or tails of tadpoles for the ChIP assay and used quantitative PCR (qPCR) with primers to measure presence of promoters of widely expressed genes from chromatin immunoprecipitated with anti-TR antibodies. These experiments take advantage of genes that are expressed in most if not all cell types, because organs or biopsies from whole animals most often are composed of multiple tissues. Direct T3 response genes expressed in restricted tissues that make up a small fraction of material in the organ may not be detectable due to low signal. For example, the matrix metalloproteinase stromelysin 3 (ST3) is a highly T3-induced gene expressed exclusively in fibroblasts, which make up a minority of the cells of the intestine. Because ST3 is not expressed in epithelial cells, which make up most of the intestine, only a small fraction of the cells containing nuclei have TR bound to the ST3 promoter, making it difficult to study TR binding to this promoter.

B. Study of Cofactors

Using the ChIP assay to detect cofactor binding to a particular region of DNA includes problems similar to those encountered when studying transcription factors, resulting from tissue and promoter specificity, and poses additional difficulties. For any one transcription factor, there is likely to be a less than 1:1 ratio of a particular cofactor. For example, a number of corepressors seem to bind well to unliganded TR, with nuclear receptor corepressor (N-CoR) and silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) as best documented (Jepsen and Rosenfeld, 2002; Yoon et al., 2003; Tomita et al., 2004). Thus, the binding of one particular corepressor presumably reduces the binding of the TR to a different corepressor. In the case of coactivators, the situation is even more complex because there are even more coactivators known to interact with TR under various *in vitro* conditions. An additional technical problem is the efficiency of crosslinking of cofactors to DNA, which will likely be less than that for transcription factors because the latter bind directly to DNA, whereas cofactors are at least one molecular interaction removed. Nevertheless, we have been able to show an increased recruitment of cofactor SRC3 to T3-responsive promoters in the intestine in the presence of T3, indicating the sensitivity and usefulness of *in vivo* ChIP assay for cofactors (Figure 11.1) (Paul et al., 2005).

C. Study of Histone Modifications

Because histones are bound directly to DNA, ChIP assay for histone modifications do not present the same problems as cofactors. Moreover, because of the relative abundance of histones, studies of histone modifications may not be as affected by tissue specificity as transcription factor-based studies (Figure 11.1c). With studying histone modifications, their relative abundance creates a potential problem, namely DNA region specificity. It is important to be able to conclude that any detectable modification is associated with the DNA region of interest. This suggests that the size of the DNA fragments cannot be too large to contain two or multiple regions

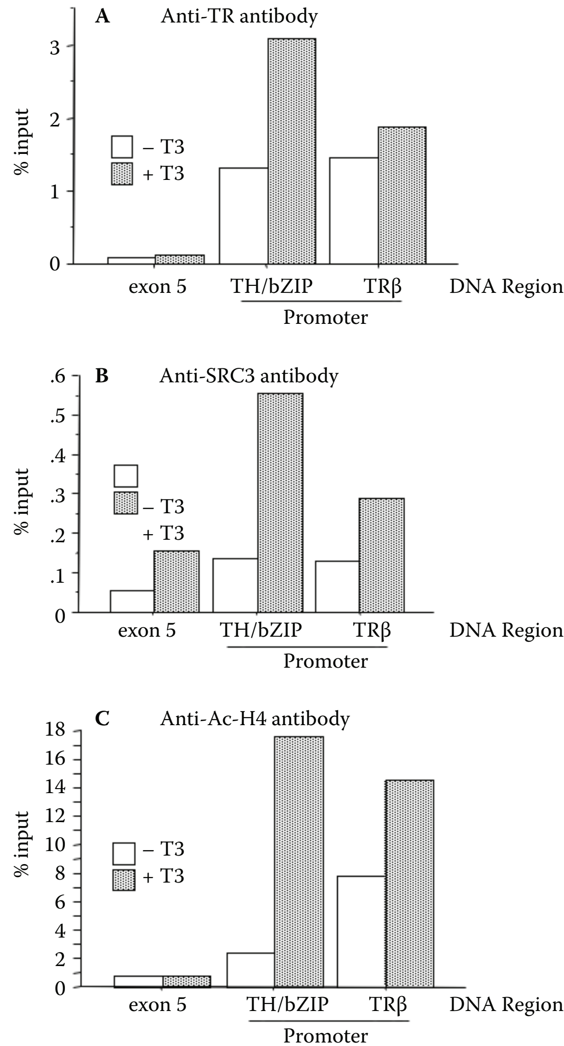
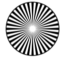
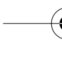
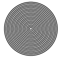


FIGURE 11.1 Using our *in vivo* model of frog metamorphosis, which totally depends on T3 and TR, we have shown hormone-dependent changes at T3-inducible promoters. Premetamorphic tadpoles before the beginning of endogenous T3 production were treated with 10 nM T3 for 2 days. ChIP assay was carried out on intestines from one to three tadpoles using antibodies for TR (a), SRC3 (b), and acetylated histone H4 (c). Notice the promoter-specific hormone-dependent changes of TR binding in (a) and hormone dependent SRC3 binding (b). Also, note the higher levels of histone acetylation in T3-treated animals. Also note that the ChIP signals, as a percentage of input DNA, were much higher for acetylated histone H4 and TR than for SRC3. This was likely due to the fact that TR and histones bind DNA directly, whereas SRC3 associate with DNA indirectly and compete for binding to TR with other coactivators. (Unpublished observations.)

of modification, so that antibodies will not recognize modified histones outside the region of interest yet pull down the region of interest.

D. Cell Culture versus Tissues, Frog vs. Mouse

The important differences between ChIP assay for cell culture and tissues is in preparation of the chromatin, whereas the subsequent immnoprecipitation steps and analysis are the same. In order to provide a snapshot of protein/DNA interactions, it is desirable to fix the cells or tissues with as little disturbance as possible. Because cells in culture are separate, they are quickly and efficiently fixed in a cell suspension with minimal disturbance. Therefore, chromatin isolation from blood cells can be similar to that for tissue culture cells. Direct fixation of tissues best preserves chromatin structure but creates problems in isolating chromatin relatively free of cellular debris. Fixation by perfusion of organs *in situ* is appropriate for tissues that are large enough and highly vascularized followed by mincing and then homogenizing in a dounce (Chaya and Zaret, 2004). Alternative methods are to isolate cell types from tissues (e.g., pancreatic islets) or to mince tissues first and then fix in formaldehyde before homogenization (Parrizas et al., 2001; Wells and Farnham, 2002).

In the current protocol, we homogenize tissues first to release cells and nuclei and then fix with formaldehyde. All these methods attempt to preserve, as closely as possible, the chromatin structure by fixing first before disrupting the nuclear membrane or altering the ionic strength found in the tissues. These methods can be used across organisms, such as frogs, mice, or tissue/cancer biopsies, but may have to be modified to accommodate different types of tissue. For example, organs high in connective tissue may require nuclear isolation first before fixation to avoid trapping the nuclei in the tissue, and embryos have little connective tissue but cannot be fixed *in situ* for lack of veins large enough for perfusion and so need to be minced first.

III. KEY ISSUES

A. Antibody

The antibody characteristics, namely, high specificity and titer, are desirable for ChIP assays be they from tissues or cell culture samples. However, these requirements may become more stringent in tissues for which it is difficult to get chromatin preparations free of cell debris that may exacerbate problems with high background from antibody cross-reactivity or in tissues where only some cell types express the gene of interest.

B. Important Controls

Reliability of results from ChIP assay in the evaluation of differences observed between samples is an important issue because of the many potential artifacts. Thus, control antibodies and DNA regions need to be included in the experimental design.

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Differences between samples may be due to one batch of chromatin being more "sticky" than another as a result of a treatment, so that even if replication of the same treatment gives similar results, the results may not necessarily reflect real binding. To control for systematic differences in chromatin between treatments, a control antibody is necessary. The control antibody can be preimmune serum or immunized to an irrelevant protein and should give results that are clearly distinguishable from the antibody of interest.

Another issue in comparing across treatments is that different time points after a treatment or different developmental stages may constitute a different collection of cell types in a developing system. If widely different developmental stages are used, the ChIP assay would be documenting differences at a developmentally regulated promoter that may be due to changes in cell types or composition, rather than studying mechanisms of gene regulation within a given cell.

Comparison across DNA regions with the same antibody needs to be done with caution because the enhanceosome may be different at different promoters and thus represent different epitopes for the antibody. Such differences can lead to differences in immunoprecipitation efficiency and antibody cross-reactivity. Also, comparing across antibodies with the same DNA region needs to be done with the caveat that different antibodies may bind with different affinities. Finally, and of course, any result needs to be repeated from multiple chromatin preparations in order to represent true biological variability.

C. Quantitative PCR vs. PCR/Gel Electrophoresis

The advantages of quantitative PCR over PCR/gel elecrophoresis are both logistical and potentially critical. Quantitative PCR measures amplification during each cycle and compares samples in the exponential phase of the reaction that can be quantitatively compared using standards. In contrast, traditional PCR detects amplified products at the endpoint of the reaction, which may or may not reflect rank order differences in input among samples due to potential saturation of reaction components. In addition, qPCR obviates the need for post-PCR analysis. In many cases, the quantity of amplicons is too low to be detected by ethidium bromide staining after agarose gel electrophoresis, so visualization of PCR results requires Southern blotting followed by chemiluminescent detection or exposure of the dried gel to film if radioactive nucleotides are included in the PCR reaction.

A more important issue is in the case where two treatments using the same antibody and DNA region give the same results. It is not easy using conventional PCR to determine whether this similarity is due to similar binding levels or because both samples are at the background level. To distinguish these possibilities, one needs to use a control DNA region where the antibody is not expected to bind and compare the results with the DNA region of interest, remembering the cautions of comparing across DNA regions discussed above. When using qPCR, the specific product is not visualized, and thus, careful controls and calibration are needed to ensure the specificity of the signal detected, especially when SYBR Green or a similar detection method is used, because nonspecifically amplified DNA will contribute to the final signal.

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IV. PROTOCOL

A. Animals and Treatment

Xenopus laevis tadpoles of different developmental stages (Nieuwkoop and Faber, 1994) were obtained from NASCO or Xenopus I, Inc. Stage 54 premetamorphic tadpoles at a density of 2–3 tadpoles per liter were treated with 10 nM T3 for 1–3 days at 18°C.

B. Reagents and Buffers

Stock materials:

37% formaldehyde solution
1 M Tris-HCl, pH 9.4 or 1 M glycine
1 M DTT, frozen in aliquots
0.2 M PMSF, in EtOH (half-life is 30 min in water)
Protease inhibitor tablet (Roche, Complete, Mini, EDTA-free)
2 ml all glass dounce homogenizer sets (Kontes Kimble) with pestles A (for initial homogenization) and B (for nuclei expulsion)
Qiaquick PCR purification kit (Qiagen)

ED: This was “Qia Quick” in Chapter 10

Working solutions:

0.6X phosphate buffered saline (PBS)
Nuclei extraction buffer (prepared fresh): 0.5% Triton X-100, 10 mM Tris-HCl, pH 7.5, 3 mM $CaCl_2$, 0.25 M sucrose, protease inhibitor tablet (1 tablet/20 µL), 1 mM DTT, and 0.2 mM PMSF
SDS lysis buffer: (Upstate, 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1)
Salmon sperm DNA/protein A agarose (Upstate)
ChIP dilution buffer: (Upstate, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl. Add 1 mM DTT, 0.4 mM PMSF, and protease inhibitor tablet [1 tablet/20 µL] just before use.)
ChIP I low salt buffer: (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 50 mM Hepes, pH 7.5; 150 mM NaCl)
ChIP II high salt buffer: (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 50 mM Hepes, pH 7.5; 500 mM NaCl)
ChIP III LiCl wash buffer: (0.25 M LiCl; 0.5% NP-40; 0.5% sodium deoxycholate; 1 mM EDTA; 10 mM Tris-HCl, pH 8.0)
TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0)
Elution buffer: 0.5% SDS, 0.1M NaHCO3 (Sigma), 25 µg/ml Proteinase K (Roche)

AU: If this is for “liter” lowercase throughout

ED: change to “one” throughout?

C. Chromatin Isolation from Tissue

1. Dissect tissue, up to 0.3 to 0.5 mg, from euthanized tadpoles and place in 1 mL nuclei extraction buffer in dounce on ice. We have tried whole

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tadpole, tail, intestine, and liver. For small intestines, flush contents using 0.6X PBS in a syringe with a 31-gauge needle, and place in 1 mL nuclei extraction buffer in dounce on ice. Pooling one to three premetamorphic tadpole organs per treatment results in sufficient chromatin concentration without overwhelming capacity of the buffer and homogenization process.

2. Crush in a dounce homogenizer with 10–15 strokes using the large clearance pestle A for initial homogenization. See Note 1.
3. Pour homogenate into 1.7 ml eppendorf tubes and add 25 µL 37% formaldehyde (1% final concentration) to crosslink proteins and DNA. Rotate tubes at room temperature for 15 min.
4. Add 100 µL 1M Tris-HCl, pH 9.5 (or glycine to 0.125 M) and continue rotating for 5 min to stop crosslinking.
5. Centrifuge at 2000 g at 4°C for 2 min.
6. Discard supernatant, resuspend pellet in 1 mL nuclei extraction buffer, and transfer to the dounce tubes on ice.
7. Rehomogenize with 5–10 strokes using pestle B. Pestle B may be difficult to use with tissues high in connective tissue, so using pestle A may be necessary.
8. Filter out the unhomogenized debris through a Falcon 100 µm cell strainer into fresh eppendorf tubes or 50 mL conical tubes and centrifuge at 2000 g at 4°C for 2 min.
9. Optional step. After filtering, the nuclei can be further purified by layering onto a 9 ml cushion of nuclei extraction buffer with 2.2 M sucrose rather than 0.25 M sucrose and then centrifuging the samples for 3 hr at 4°C at 55,000 rpm using an HB4 rotor and Sorvall Ultracentrifuge (Damjanovski et al., 2002). See Note 2.
10. Resuspend pellet in 200–300 µl SDS lysis buffer on ice.
11. Shear the chromatin to approximately 200 to 1000 bp fragments using a sonicator, while keeping the samples on ice throughout the process to avoid overheating. See Note 3.
12. Centrifuge the sonicated solution at 14,000 rpm in an eppendorf microfuge for 10 min at 4°C. Transfer the supernatant to fresh tubes and quantitate the DNA by measuring the absorbance at A260 using a spectrophotometer. See Note 4.
13. At this stage, the chromatin can be aliquotted, snap frozen in liquid nitrogen, and stored at –80°C. We have stored aliquots for many weeks without affecting the results.

AU: Need to define?

D. Immunoprecipitation and DNA Purification

1. Remove a frozen aliquot and adjust the DNA to 100 ng/µl using the SDS lysis buffer. Then, dilute the samples to a final concentration of 10 ng/µl (down to 3–5 ng/µl also works) using the ChIP dilution buffer so the total volume is enough for 500 µL per immunoprecipitation. (For instance, when using three antibodies, take 200 µl of 100 ng/µl sample in SDS lysis

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buffer and add 1800 µL of ChIP dilution buffer in 5 mL Falcon tubes so there is enough volume for each antibody and 20 µL for the input sample.)

2. Preclear the chromatin before dividing into separate tubes for antibodies using 60 µl of slurry of salmon sperm DNA/protein A agarose per 1 mL of chromatin solution for 30 min with rotation at 4°C to reduce background or nonspecific DNA/protein binding to the agarose beads. Use salmon sperm DNA/protein G agarose if using monoclonal antibodies.
3. Pellet the agarose beads by centrifugation at 1000 g for 2 min at 4°C.
4. Set up immunoprecipitation: Add 10–60 µl of slurry of salmon sperm DNA/protein A agarose into 1.7 mL eppendorf tubes, enough tubes for all antibodies and chromatin samples. Then, for each chromatin sample, add 500 µL of precleared sample into the same number of tubes as antibodies. (Between 0.5 and 1.0 ml of the chromatin solution with a total DNA concentration ranging from 3.5 to 10.0 µg can be used per immunoprecipitation.) Add the appropriate amount of antibody to each tube with 500 µL of sample and agarose. (We use between 5 and 40 µL of antibody per tube. Both antisera as well as purified antibodies can be used. See Note 5. A preimmune serum or irrelevant antibody should be used as a negative control. A no-antibody control can be used to troubleshoot high background.)
5. Pipet 20 µL of each chromatin sample into another 1.7 mL eppendorf tube for input controls and store at 4°C until de-crosslinking.
6. Incubate immunoprecipitation samples with rotation from 4 hr to overnight at 4°C.
7. After incubation, pellet the beads at 1000 g for 2 min at 4°C, remove supernant (as much as possible to avoid high background), and add 1 mL of ChIP I.
8. Rotate for 5–15 min at 4°C, then repeat wash (step 7) with 1 ml of ChIP II, III, and TE buffers.
9. After last wash with TE buffer and removal of supernatant, add 100 µL of elution buffer to the beads in each tube and rotate at 65ºC for 6 hr to overnight to reverse crosslinks. Do not forget to include input samples at this step.
10. Purify DNA by phenol/chloroform extraction or using the Qiaquick PCR purification kit (Qiagen).
11. Resuspend (for phenol/chloroform extraction) or elute DNA (for the Qiagen kit) in 40 µl of water or EB buffer (Qiagen, 10 mM Tris-HCl, pH 8.5); 4 µL can be used for each PCR reaction.

E. PCR/Gel Analysis and Quantitative PCR

Conventional PCR, using α-^{32}P-dNTP followed by polyacrylamide gel electrophoresis, or quantitative PCR can be employed for the DNA amplification and detection. For conventional PCR, for example, assemble a 20 µl reaction on ice as follows (the conditions for each primer set have to be determined empirically):

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10X Taq buffer free of Mg	2 µl
25 mM $MgCl_2$	2 µl
Primer A	25 ng (0.1 µl)
Primer B	25 ng (0.1 µl)
25 mM dNTPs	0.1 µl
Taq polymerase (5 µ/µl, promega)	0.1 µl
α-^{32}P-dCTP or ^{32}P -dATP	0.1 µl
H_2	11.5 µl

PCR cycling: 95ºC for 30 sec, 62ºC for 30 sec, 72ºC for 30 sec, 20–37 cycles depending on the intensity of signals, then 72ºC for 5 min.

After PCR, add 4 µl of 5X DNA loading buffer to each tube. Separate on a 6% native polyacrylamide gel using 1X TBE. Load 5–8 µl of the PCR reaction per lane. Run the gel at 300 V for 40 min to 1 hr. Dry the gel on Whatman No. 3 filter paper and visualize by autoradiography. Care should be taken to avoid saturation of the PCR (especially for input DNA control) and autoradiography exposure so that any potential differences between samples can be identified, especially the input samples that are used to normalize (visually or using densitometry) the immunoprecipitation samples. Thus, it is worthwhile to try different numbers of PCR cycles to avoid saturation.

Quantitative PCR is used routinely in our lab for analysis of the ChIP assay. In our experience, qPCR yields the same rank order differences between samples as conventional PCR. We use promoter-specific primers and FAM (6-carboxyfluorescein)-labeled *Taq*-man probes, which are much preferred and increase the specificity of the reaction compared to SYBR Green, on an ABI 7000 (Applied Biosystems). Quantitation with *Taq*-man probes involves a fluorescent moiety attached to the probe that is quenched in the unbound state and gives a fluorescent signal when bound to the proper sequence in the PCR product. Thus, as the PCR cycle number increases, more probes are in the bound state to give more fluorescent signal. Each assay includes standards, a no-template control, a control sample, and the input and experimental samples. A standard curve is generated using six threefold serial dilutions from a standard (concentrated ChIP input DNA, enough made and frozen in aliquots, to last at least across all the assays in the experiment so different qPCR runs can be compared). The theoretical slope for the standard curve is –3.32. The initial concentration of the standard and dilutions are chosen to encompass the input and experimental samples so they fall within the standard curve. The no-template control is pure water to monitor PCR product contamination. The control sample is a known amount of DNA, e.g., 0.1 µg/µl tadpole genomic DNA, and is used to assess the consistency of the standard curve calculated from the standards across qPCR runs. The values for the input and experimental samples are calculated from the standard curve, and then percentage input is calculated for each experimental sample from the corresponding input sample.

We test three kinds of DNA regions in qPCR experiments. For us, the promoters of interest are thyroid hormone-regulated promoters containing T3 response elements (TREs), which are the regions that we amplify. We use two negative controls. First,

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a control promoter lacking a TRE is not expected to bind the transcription factor of interest, in our case thyroid hormone receptor, and is not expected to change across treatments. Second, a nonpromoter region at least 3000 base pairs away from the promoter region of interest, such as a downstream exon controlled by the promoter of interest, is used to identify potential high background from poor sonication, nonspecific antibody binding, incomplete washing after immunoprecipitation, or nonspecific binding from too much antibody. Also, by comparing the signal from this nonpromoter control region to that from the experimental DNA region, we can determine whether the antibody gives specific signal in the experimental DNA region (the TRE region in our case).

V. NOTES

Note 1: Examination using a hemocytometer after this step reveals debris from disrupted cells, intact nuclei, and few intact cells.

We have examined the homogenate after this step by treating the preparation with DAPI and examining an aliquot on a hemocytometer under a fluorescent compound microscope. We found large amounts of debris and that most cells are disrupted. Most of the cell debris is removed by washing in nuclei extraction buffer, followed by rehomogenization and filtration; most of the cell debris is removed, resulting in relatively pure nuclei. After the optional centrifugation through the high-sucrose buffer, the nuclei are difficult to distinguish from debris because they are shrunken and misshapen from the high ionic strength of the sucrose buffer, so that it is not clear if this step improves purity.

Note 2: The previous protocol purified the nuclei through a high-sucrose buffer. This step is not necessary in our hands. In addition, in the previous protocol, DNA-protein crosslinking was done after nuclei isolation. To better preserve native DNA-protein interactions, we have tried to add formaldehyde during tissue homogenization or after homogenization but before nuclei isolation. In our hands, they all produced qualitatively similar ChIP results as the previous method, although we have noticed a higher signal using antibodies against modified histones by skipping the high-sucrose step.

ED: Not an en dash?

Note 3: The sonication condition should be optimized depending on the sonicator used. To optimize the shearing conditions, set up a pilot experiment with different extents of sonication. Check the levels of sonication on a 1% agarose gel after de-crosslinking and DNA purification to ensure ~200–1000 base pair size. Immerse the microtip in the solution in a 1.7 mL eppendorf tube, keeping the tip quite close to the bottom and avoiding the walls of the tube. Lower sonication volumes lead to a higher shearing efficiency, so that changing the volume requires reoptimization. Avoid trapping air bubbles and emulsifying the sample during sonication, as this can compromise the efficiency of sonication. Under our conditions with a Branson sonifier 450 set at 30% duty cycle and output control 2, 10–12 cycles of 10-sec pulses with 10-sec cooling between pulses using a stepped microtip yielded optimal results.

Some workers include a restriction enzyme step as an additional means to fragment the DNA to reduce the proportion of larger sized fragments 1–2 kilobases

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(kb) in the chromatin mixture (Chaya and Zaret, 2004). This step is important for experiments where DNA regions of interest are relatively close to each other (within 2 kb) or for when the immunoprecipitated chromatin will be subsequently used on a microarray to identify unknown promoter targets. We have not tried to incorporate this step because our experiments have thus far involved only comparisons of transcription factor and cofactor binding to known promoters very distant from each other.

Note 4: If RNA concentration is too high and interferes with the DNA measurement, treat samples with RNAse, purify DNA, and then measure. We generally have very little RNA as evidenced by the lack of transfer RNAs in the gel for checking sonicated DNA. DNA yields vary depending upon tissue, where we usually get between 45 and 90 μg chromatin from tails and 60 and 180 μg from intestines with 0.3–0.5 mg of starting material.

Note 5: A titration needs to be performed for each antibody to identify the appropriate concentration. For example, in a pilot experiment, prepare six tubes with 500 μL of sample and add 5, 10, 15, 20, 40 μL to the first five tubes and add 40 μL of preimmune serum or an irrelevant antibody to the sixth tube. Complete the ChIP assay and choose the antibody amount that gives the highest signal from the experimental DNA region and the least background from a control DNA region.

VI. SHORT VERSION OF PROTOCOL

Chromatin isolation from tissue:

1. Place dissected tissue in 1 mL nuclei extraction buffer in dounce on ice.
2. Crush with 10–15 strokes using pestle A.
3. Transfer to 1.7 ml tubes, add 25 uL 37% formaldehyde, and rotate at room temperature for 15 min.
4. Add 100 μL 1 M Tris-HCl, pH 9.5 and rotate for another 5 min.
5. Centrifuge at 2000 g at 4°C for 2 min.
6. Resuspend pellet in 1 ml of nuclei extraction buffer, transfer to the dounce tubes on ice.
7. Rehomogenize with 5–10 strokes.
8. Filter through a Falcon 100 μm cell strainer and recentrifuge.
9. Resuspend pellet in 200–300 μl SDS lysis buffer on ice.
10. Sonicate.
11. Centrifuge at 14,000 rpm for 10 min at 4°C. Transfer the supernatant to fresh tubes and quantitate DNA.
12. Make frozen aliquots.

Immunoprecipitation and DNA purification:

1. Adjust the DNA to 100 ng/μl using the SDS lysis buffer. Then, dilute samples to 10 ng/μl with ChIP dilution buffer.
2. Preclear the chromatin using 60 μl of slurry of salmon sperm DNA/protein A agarose per 1 mL of chromatin solution for 30 min with rotation at 4°C.

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3. Pellet the agarose beads by centrifugation at 1000 g for 2 min at 4°C.
4. Immunoprecipitation: Add 10–60 µl of slurry of salmon sperm DNA/protein A agarose to appropriate number of empty tubes. Aliquot 500 µL of precleared chromatin sample into one tube for each antibody. Add antibody.
5. Pipet 20 µL into another 1.7 mL eppendorf tube for input controls.
6. Incubate the samples with rotation from 4 hr to overnight at 4°C.
7. After incubation, pellet the beads at 1000 g for 2 min at 4°C, remove supernatant, and add 1 mL of ChIP I.
8. Rotate for 5–15 min at 4°C, then repeat wash (step 7) with 1 ml of ChIP II, III, and TE buffers.
9. After last wash, add 100 µL of elution buffer and rotate at 65ºC for 6 hr to overnight. Do not forget to include input samples at this step.
10. Purify DNA by phenol/chloroform extraction or using the Qiaquick PCR purification kit (Qiagen).
11. Resuspend (for phenol/chloroform extraction) or elute DNA (for the Qiagen kit) in 40 µl of water or EB buffer (Qiagen, 10 mM Tris-HCl, pH 8.5).

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REFERENCES

Buchholz, D.R., Hsia, S.-C.V., Fu, L., Shi, Y.-B., 2003. A dominant negative thyroid hormone receptor blocks amphibian metamorphosis by retaining corepressors at target genes. Mol. Cell. Biol. 23, 6750–6758.

Buchholz, D.R., Tomita, A., Fu, L., Paul, B.D., Shi, Y.-B., 2004. Transgenic analysis reveals that thyroid hormone receptor is sufficient to mediate the thyroid hormone signal in frog metamorphosis. Mol. Cell. Biol. 24, 9026–9037.

Chaya, D., Zaret, K.S., 2004. Sequential chromatin immunoprecipitation from animal tissues. Methods Enzymol. 376, 361–372.

Damjanovski, S., Sachs, L.M., Shi, Y.-B., 2002. Function of thyroid hormone receptors during amphibian metamorphosis. Methods Mol. Biol. 202, 153–176.

Das, P.M., Ramachandran, K., vanWert, J., Singal, R., 2004. Chromatin immunoprecipitation assay. Biotechniques 37, 961–969.

Farnham, P.J., Weinmann, A.S., 2002. Identification of unknown target genes of human transcription factors using chromatin immunoprecipitation. Methods 26, 37–47.

Jepsen, K., Rosenfeld, M.G., 2002. Biological roles and mechanistic actions of co-repressor complexes. J. Cell Sci. 115, 689–698.

Kuo, M.-H., Allis, C.D., 1999. In vivo cross-linking and immunoprecipitation for studying dynamic protein:DNA associations in a chromatin environment. Methods 19, 425–433.

Nieuwkoop, P.D., Faber, J., 1994. Normal table of Xenopus laevis (Daudin). Garland Publishing, Inc., New York, p. 252.

Parrizas, M., Maestro, M.A., Boj, S.F., Paniagua, A., Casamitjana, R., Gomis, R., Rivera, F., Ferrer, J., 2001. Hepatic nuclear factor 1-alpha directs nucleosomal hyperacetylation to its tissue-specific transcriptional targets. Mol. Cell. Biol. 21, 3234–3243.

Paul, B.D., Fu, L., Buchholz, D.R., Shi, Y.-B., 2005. Coactivator recruitment is essential for liganded thyroid hormone receptor to initiate amphibian metamorphosis. Mol. Cell. Biol. in press.

3165_book.fm Page 271 Monday, April 24, 2006 3:00 PM

Sachs, L.M., Damjanovski, S., Jones, P.L., Li, Q., Amano, T., Ueda, S., Shi, Y.-B., Ishizuya-Oka, A., 2000. Dual functions of thyroid hormone receptors during *Xenopus* development. Comparative Biochemistry and Physiology, Part B 126, 199–211.

Sachs, L.M., Jones, P.L., Havis, E., Rouse, N., Demeneix, B.A., Shi, Y.-B., 2002. Nuclear receptor corepressor recruitment by unliganded thyroid hormone receptor in gene repression during *Xenopus laevis* development. Mol. Cell. Biol. 22, 8527–8538.

Sachs, L.M., Shi, Y.-B., 2000. Targeted chromatin binding and histone acetylation in vivo by thyroid hormone receptor during amphibian development. Proceedings of the National Academy of Sciences 97, 13138–13143.

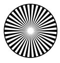
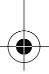
Shi, Y.-B., 1999. Amphibian metamorphosis: From morphology to molecular biology. John Wiley and Sons, Inc., New York, pp. 1–288.

Spencer, V.A., Sun, J.-M., Li, L., Davie, J.R., 2003. Chromatin immunoprecipitation: a tool for studying histone acetylation and transcription factor binding. Methods 31, 67–75.

Tomita, A., Buchholz, D.R., Shi, Y.-B., 2004. Recruitment of N-CoR/SMRT-TBLR1 corepressor complexes by unliganded thyroid hormone receptor for gene repression during frog development. Mol. Cell. Biol. 24, 3337–3346.

Wells, J., Farnham, P.J., 2002. Characterizing transcription factor binding sites using formaldehyde crosslinking and immunoprecipitation. Methods 26, 48–56.

Yoon, H., Chan, D., Huang, Z., Li, J., Fondell, J., Qin, J., Wong, J.M., 2003. Purification and functional characterization of the human N-CoR complex: the roles of HDAC3, TBL1 and TBLR1. EMBO J. 22, 1336–1346.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