Chromatin immunoprecipitation on native chromatin from cells and tissues (PROT22)

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Introduction

In cells and tissues, the histone proteins that constitute the nucleosomes can present multiple post-translational modifications (Luger & Richmond, 1998), such as lysine acetylation, lysine and arginine methylation, serine phosphorylation, and lysine ubiquitination. On their own, or in combination, these covalent modifications on the core histones are thought to play essential roles in chromatin organisation and gene expression in eukaryotes (Hebbes *et al*., 1994; O'Neill & Turner, 1995; Grunstein, 1998; Turner, 2000; Jenuwein & Allis, 2001). Importantly, patterns of histone modifications may be somatically conserved and can, thereby, maintain locus-specific repression/activity in defined lineages, or throughout development. Indirect immuno-fluorescence studies on cultured cells have been pivotal in unravelling the roles of histone modifications. These studies have been highly informative on the functions of specific histone modifications in, for instance, pericentric chromatin condensation (Peters, *et al*., 2001; Maison, *et al*., 2002) and X-chromosome inactivation (Heard *et al*., 2001, Boggs *et al*., 2002, Peters *et al*., 2002) in mammals (H3 and H4 deacetylation, and H3-K9 methylation). However, particularly in mammalian model systems, it remains poorly understood how histone modifications are organised at specific chromosomal regions and genes. To address in detail what happens at specific sites in vivo, chromatin immuno-precipitation (ChIP) is the method of choice. Here, we describe how ChIP can be performed on native chromatin extracted from cells, or tissues, to analyse histone methylation and acetylation at specific chromosomal sites. In addition, we present different PCR-based methods that allow the analysis of a locus of interest in chromatin precipitated with antibodies to specific histone marks (see figure 1 for an overview of the described procedures). Should you require a literature reference, please, quote an earlier paper by our group, where these methodologies were originally described (Umlauf *et al.*, 2003).

Background information

Chromatin immuno-precipitation

Chromatin immuno-precipitation (ChIP) is performed by incubation of fractionated chromatin (input chromatin) with an antiserum directed against a chosen histone modification. As a general rule, there are two ways to obtain 'input' chromatin. Several groups in the field prepare 'cross-linked chromatin', for example, by photochemical cross-linking, or by chemically cross-linking proteins and DNA with specific substances, such as formaldehyde (Orlando, 2000). Formaldehyde cross-linking is

particularly suitable for ChIP studies on histone modifications. However, usually only a small fraction of the chromatin is precipitated, and it relies on random shearing, which does not always produce small-enough chromatin fragments at the regions of interest. For this reason and to conduct experiments on fresh and frozen tissues, we and others have preferred to make use of 'native chromatin' (O'Neill & Turner, 1995; Gregory *et al*., 2001). In these protocols (Figure 1), the chromatin is fractionated by incubation of purified nuclei with micrococcal nuclease (MNase), an enzyme that cleaves preferentially the linker DNA between the nucleosomes (Drew, 1984). Specifically, by performing partial digestions with MNase, it is possible to obtain native chromatin fragments of on average one to five nucleosomes in length (Figure 2). These oligo-nucleosome fragments are purified from the nuclei and are then used to perform ChIP. The choice of native, MNase-fractionated, chromatin as the input material for ChIP is advantageous, because the epitopes, recognised by the antibody, remain intact during the chromatin preparation. As a consequence, native chromatin tends to give higher levels of precipitation for a specific histone modification than formaldehyde cross-linked chromatin (Goto *et al*., 2003). The ChIP protocol presented below describes in detail how to prepare and immuno-precipitate native chromatin. This protocol was adapted from methodology originally described by O'Neill and Turner (1995), and allows ChIP to be performed not only on chromatin from cultured cells, but also on freshly dissected and frozen tissues. It is adapted to the analysis of histone methylation and acetylation.

Although ChIP is presently the best-available methodology to analyse histone modification at specific chromosomal loci, it has several limitations. Firstly, unlike DNA methylation studies, ChIP does not allow analysis of histone modifications in individual cells, or on individual chromosomes. ChIP studies are always performed on populations of (cultured) cells, or on tissue samples comprising many cells. Although sequential precipitations with different antisera could be attempted, this method seems also not suitable to determine whether there are specific combinations of covalent modifications on individual histones at a given locus. Again, this is because many cells are used for chromatin purification and ChIP, and chromatin is usually fractionated into fragments that comprise multiple nucleosomes. Lastly, it should be noted that quantification of the levels of histone modifications at specific chromosomal loci is difficult to obtain by ChIP, because levels of precipitation do not depend simply on the local abundance of the modification studied. They also depend, to a great extent, on the quality of the prepared chromatin, and on how the chromatin was prepared (eg, native versus cross-linked), on the quality, specificity and concentration of the antiserum used for ChIP, and on the global abundance of the histone modification that is being studied. The later limitation of the technology is discussed in more detail in the footnotes below.

PCR-based analysis of precipitated chromatin

After ChIP, precipitated chromatin fractions are analysed by optical density (OD) reading and agarose gel electrophoresis to assess the quantity and quality of the precipitated chromatin. DNA is then extracted to allow analysis of the chromosomal site(s) of interest. In several earlier studies on locus-specific histone modifications, regions of interest were analysed by Southern hybridisation of slot blots (O'Neill & Turner, 1995; Hebbes *et al*., 1988). More recently, however, quantitative amplification by polymerase chain reaction (PCR) has become the method of choice. Different PCR-based approaches can be used to determine how much DNA is precipitated at a site of interest. Although 'real-time' PCR amplification is often the preferred technique to quantify amounts of chromatin precipitated at specific loci, 'Duplex PCR amplification', which is the co-amplification of a fragment from the region of interest and a control fragment (e.g. the actin gene, or the tubulin gene), can also be used. Duplex PCR amplification has been successful in studies on the *S. pombe* mating-type loci and for analysis of imprinted mammalian genes (Figure 3), as it allows to detect relative levels of specific histone modifications along chromosomal domains (Gregory *et al*., 2001; Norma *et al*., 2001). Alternatively, in particular for allelic studies on dosage-compensation mechanisms, or on genomic imprinting in mammals, Single-Strand Conformation Polymorphisms (SSCP, Figure 4) (Orita *et al*., 1989; Gregory & Feil, 1999), or similar strategies, such as 'Hot Stop PCR' (Uejima *et al*., 2000), can be put to use to differentiate PCR products that represent the silent allele from those amplified from the active allele (Goto *et al*., 2003; Fournier *et al*., 2002). These different PCR-based approaches will be described in detail in the section on quantitative PCR analysis of precipitation chromatin.

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Procedure

Nuclei Preparation from Tissues and Cells

To prevent chromatin degradation by endogenous nucleases and to keep the chromatin intact, all steps of the nuclei purification procedure should be performed on ice, or at 4°C (e.g. pre-cool the centrifuge rotors). In addition, dedicate one set of pipettes only for the preparation of nuclei, chromatin and ChIP analysis, to avoid contamination with non-genomic DNA (plasmids, PCR products, etc.).

Purification of Nuclei from Tissues

1. Dissect tissue (do not use more than 0.2g in total) and rinse it in cold PBS (see note 1 and note 2);
2. Homogenise tissue in a pre-chilled glass homogenizer with 5-10mL of ice-cold Buffer I, until no clumps of cells persist

(about 10-20 strokes). Filter the cell suspension through 4 layers of muslin cheese cloth that have been moistened beforehand with 2mL of Buffer I;
3. Transfer the cell suspension to a 14mL polypropylene tube and spin cells down in a swing-out rotor (at 6000 g for 10 minutes, at 4°C);
4. Pour off the supernatant and re-suspend the cells in 2mL of ice-cold Buffer I. Then add 2 mL of ice-cold Buffer II (see note 3 and note 4), mix gently, and place on ice for 10 minutes.
5. Prepare two new 14mL polypropylene tubes containing each 8mL of ice-cold Buffer III. Carefully layer 2mL of each cell suspension (from step 4) on each 8mL sucrose cushion. Cover the tubes with a piece of Parafilm®;
6. Centrifuge in a pre-chilled swing-out rotor, at 10,000 g, for 20 minutes at 4°C. During this centrifugation step, the nuclei will form a pellet on the bottom of the tube, whereas the cytoplasmic components will remain in the top layer (see note 5);
7. Carefully take off the supernatant with a Pasteur pipette. This is a critical step, as the top solution (which contains the detergent IGEPAL CA-630®) should not come into contact with the nuclear pellet at the bottom of the tube. One way to achieve this, is to remove the supernatant in about three times, each time changing the Pasteur pipette (see note 5);
8. Re-suspend the nuclei pellet into 1mL of MNase digestion buffer and put on ice. Nuclei can, at this point, be counted by using a microscope slide for counting cells. The number of nuclei obtained per gram of tissue varies between different tissue types. For liver, for example, the above protocol yields ~$2x10^9$ nuclei/gram of tissue. (Frozen tissues can be used for nuclei preparation as well (see note 6, note 7 and comment 1).

Nuclei Preparation from Cultured Cells

Culture $5x10^7$ to $5x10^8$ cells in the appropriate culture medium. Ensure that cells are not grown beyond semi-confluency.

1. Rinse cells in PBS, add 2mL of trypsin solution (for adhering cells only) and incubate at 37°C;
2. When trypsination is complete, stop the reaction by adding 5mL of culture medium to the cells;
3. Divide the cell suspension amongst two polypropylene 14mL tubes and spin cells down in a swing-out rotor (4000 g, 5 minutes at 4°C);
4. Pour off the supernatant and re-suspend the cells in 2mL of ice-cold Buffer I. Then add 2mL of ice-cold Buffer II (see note 3 and note 4), mix gently, and place on ice for 10 minutes;
5. Prepare two new 14mL polypropylene tubes containing each 8 mL of ice-cold Buffer III. Carefully layer 2mL of each cell suspension (from step 4) on each 8mL sucrose cushion. Cover the tubes with a piece of Parafilm®;
6. Centrifuge in a pre-chilled swing-out rotor, at 10,000 g, for 20 minutes at 4°C. During this centrifugation step, the nuclei will form a pellet on the bottom of the tube, whereas the cytoplasmic components will remain in the top layer (see note 5);
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MNase Fractionation and Purification of Chromatin

Dialysis tubing needs to be prepared before starting the purification of chromatin. Several batches can be prepared and stored at 4°C for several weeks.

Preparation of Dialysis Tubing

1. Cut the tubing into pieces of convenient length (10-20cm);
2. Boil the tubes for 10 minutes in 0.5L of tubing preparation solution I;
3. Rinse the tubes twice in distilled water;
4. Boil the tubes for 10 minutes in 0.5L of tubing preparation solution II;
5. Allow the tubes to cool down and store them in tubing preparation solution II at 4°C. Ensure that the tubes are entirely submerged;
6. Before use, wash the tubing twice inside and out with distilled water.

MNase Fractionation

1. Purified nuclei (as described the section titled Nuclei Preparation from Tissues and Cells) are re-suspended in 1mL of ice-cold MNase digestion buffer and placed on ice;
2. Aliquot the suspension in two 1.5mL Eppendorf tubes (500µl of re-suspended nuclei in each tube);
3. Add 1µl of MNase enzyme to each tube and mix gently;
4. Incubate the two tubes in a 37° water-bath for 6 and 9 minutes, respectively;
5. Add 20µl of stop solution;
6. Chill on ice.

Recovery of Soluble Chromatin Fractions

1. Centrifuge the 1.5mL tubes with the MNase-digested nuclei at 10,000 rpm (4°C) for 10 minutes to pellet the nuclei;
2. Transfer the supernatant into another 1.5mL tube and store at 4°C. This supernatant contains the first soluble fraction of chromatin, S1, which comprises small fragments only. Do not discard the pellet;
3. Carefully re-suspend the pellet in 500µl of dialysis buffer;
4. Close one side of the washed dialysis tubing (see section titled Purification of Nuclei from Tissues, step 6) with a universal closure clamp. Transfer the 500µL of re-suspended nuclei into the dialysis tube and close the other side with a second clamp;
5. Submerge the tube for 12-16 hours in 1-2L of dialysis buffer. Dialysis is performed at 4°C, in a beaker with constant mild stirring using a magnetic stirrer;
6. Transfer the dialysed nuclei into a 1.5mL Eppendorf tube;
7. Centrifuge for 10 minutes at 10,000 rpm, at 4°C, in a micro-centrifuge;
8. Transfer the supernatant in a new 1.5mL Eppendorf tube and store at 4°C. This is the second soluble chromatin fraction, S2, comprising larger fragments of chromatin, that were removed from the nuclei during the dialysis;
9. Re-suspend the pellet, which is the chromatin fraction P, in 50µl of dialysis buffer and store at 4°C.

Quality Control of Chromatin

1. Take the Optical Density (OD) of each fraction at 260nm;
2. In separate 1.5mL Eppendorf tubes, put 0.5µg of each fraction (S1, S2, and P);
3. Add 2µL of loading buffer, and 1µL of 10% SDS. Adjust the volume to 10µL and mix gently;
4. Load the samples onto a standard 1% (w/v) agarose gel (of about 10-15cm in length) in 1x TBE electrophoresis buffer, with the 100bp DNA ladder as a size control. Let the samples migrate at 2-3V/cm until the fastest blue marker in the loading buffer has migrated till about half way the gel;
5. Stain the gel for 30 minutes in a tray with 500mL of distilled H_2O to which 10µg of Ethidium Bromide have been added;
6. Remove background staining from the gel, by rinsing for 15 minutes in distilled H_2O;
7. Control the size of the chromatin fragments under an UV lamp, and take a photograph (see figure 2 for an example of typical S1 and S2 fractions). The pellet fraction, P, consists of chromatin fragments that are longer than 5 nucleosomes in length.

Chromatin Immuno-precipitation

When an antiserum is used for the first time, it should be important to verify that the histone modification, it is directed against, has become enriched in the antibody-bound fraction. This can be done by purifying the histone proteins from this fraction followed by electrophoresis through acid-urea-triton gels (O'Neill & Turner, 1995; Bonner *et al*., 1980). After electrophoresis, proteins are Western blotted to nylon filters, which are immuno-stained with the antiserum, following standard procedures (Sambrook & Russell, 2001). An example of this procedure (Bonner *et al*., 1980) is presented by Gregory *et al*. (2002), relative to a study on histone acetylation in ES cells and fibroblasts.

Incubation of Chromatin with Antiserum

1. Mix 10-20µg of the first (S1) and 10-20µg of the second (S2) chromatin fractions in a 1.5mL Eppendorf tube (see note 8);
2. Complete the volume to 1mL with ChIP incubation buffer;
3. Add 5-10µg of the antibody of choice;
4. Close the tubes and seal the lids with some Parafilm®. Rotate the tubes at 20-30 rpm for 12-16 hours at 4°C. During this incubation time the antibodies will bind to their specific epitopes;
5. Meanwhile, aliquots of Protein-A (G) Sepharose (see following section) can be prepared for the extraction of the immuno-precipitated chromatin (see section titled Extration of Immuno-precipitated Chromatin with Protein-a (G) Sepharose).

Preparation of Protein-A (G) Sepharose (see note 8 and note 9)

1. Weigh 0.25g of Protein-A (G) Sepharose beads into a 14mL polypropylene tube;
2. Add 1mL of sterile water to moisten the beads;
3. Wash with 10mL of sterile water and mix;
4. Centrifuge for 3 minutes at 1500 g in a swing-out rotor and discard the supernatant;
5. Repeat steps 3 and 4 four times;
6. Add 1mL of sterile water and re-suspend the beads;
7. Distribute 100µL aliquots in ten 1.5mL Eppendorf tubes. Store these aliquots at 4°C. They can be used for the extraction of antibody-bound chromatin from ChIP experiments.

Extraction of Immuno-precipitated Chromatin with Protein-A (G) Sepharose

1. Add 50µl of protein-A Sepharose to each tube after the immuno-precipitation (step 4 of incubation of chromatin with antiserum) (see note 9);
2. Let the tubes rotate at 20-30 rpm, for 4 hours, at 4°C;
3. Centrifuge at 1500 g in a swing-out rotor for 3 minutes;
4. Transfer the supernatant to a 2mL Eppendorf tube. This fraction contains the chromatin which did not link the antibody: the unbound fraction. To be stored on ice;
5. Re-suspend the Sepharose beads in 1mL of Washing buffer A;
6. Transfer the re-suspended beads to a 15mL Falcon tube;
7. Complete to 10 mL with Washing buffer A. Mix briefly;
8. Centrifuge for 3 minutes at 1500 g (4°C) in a swing-out rotor. Carefully discard the supernatant;
9. Re-suspend beads in 10mL of Washing buffer B. Briefly mix;
10. Centrifuge for 3 minutes at 1500 g (4°C) in a swing-out rotor. Carefully discard the supernatant;
11. Re-suspend the Sepharose beads in 10mL of Washing buffer C;
12. Centrifuge for 3 minutes at 1500 g (4°C) in a swing-out rotor. Carefully discard the supernatant;
13. To elute the chromatin, re-suspend the Sepharose beads in 500µl of Elution buffer and transfer to a 1.5mL Eppendorf tube;
14. Incubate for ~30 minutes at room temperature on a rotating wheel at 20-30 rpm. After this incubation, centrifuge for 3 minutes at 1500 rpm in a bench-top micro-centrifuge;
15. Carefully transfer the supernatant into a 2mL Eppendorf tube. The supernatant contains the chromatin eluted from the Sepharose beads (i.e. the bound fraction). To be stored on ice.

DNA extraction and Assessment of Precipitated Chromatin

DNA Extraction from Precipitated Chromatin

1. Add 500µL of phenol:chloroform:iso-amylalcohol 25:24:1 (v/v/v) to bound (step 15 of previous section) and unbound (step 4 previous section) fractions;
2. Vortex 30 seconds;
3. Centrifuge at 13,000 rpm (~15,000 g) for 15 minutes in a bench-top micro-centrifuge;
4. Carefully transfer the upper, aqueous, phase to another 2mL Eppendorf tube;
5. Add NaCl to a final concentration of 250 mM;
6. Add 20-40µg of glycogen and mix. Since the DNA concentration in the bound fraction is usually low, we recommend the use of glycogen as co-precipitator. This step is not necessary for the 'unbound' fraction;
7. Add 1 volume of iso-propanol;
8. Mix and store at -80°C for at least 2 hours;
9. Centrifuge at 13,000 rpm in a micro-centrifuge for 15 minutes. Carefully discard the supernatants;
10. Rinse the pellets with 1mL of 70 % (v/v) ethanol;
11. Centrifuge at 13,000 rpm for 5 minutes in a micro-centrifuge. Carefully discard the supernatants;
12. Dry pellets for 5-10 minutes at room temperature and re-suspend them in 10-50µl of 1X TE buffer.

Assessment of Precipitated Chromatin

Measure the OD_{260nm}
of each sample in order to calculate how much to use as template for the subsequent PCR amplification. The ratio between the bound fraction DNA versus total starting material (corresponding to the bound and unbound fractions together; this value was obtained in step 1 of section titled Quality Control of Chromatin) indicates the efficiency of the ChIP assay, as it represents the percentage of immuno-precipitated chromatin. In a standard analysis of histone modifications no more than 15% of the input native chromatin should be precipitated. However, the percentage of overall precipitation depends on the nature and the abundance of the histone modification, and on the characteristics and concentration of the antibody used (see note 10).

Quantitative PCR Analysis of Precipitated Chromatin

Real Time PCR Amplification

In real-time PCR, each amplification is run in duplicate to control for PCR variations. The standard curve is constructed from the log-linear amplification phase using external DNA controls (we use four different concentrations of a control mouse genomic DNA). This curve will then be used to calculate the amount of target DNA in the starting material. To be able to compare regions within the same ChIP, results are presented as the percentage of the input chromatin that is precipitated at the region of interest. The following steps are according to the standard protocol provided with the Quantitect SYBR Green PCR kit (Qiagen).

1. Put 20-50ng of template DNA into a capillary specific for the real-time PCR machine;
2. Add forward and reverse primers to a final concentration of 0.4µM/each;
3. Add 9µl of 2x QuantiTect SYBR Green PCR mixture;
4. Complete to 18µl with sterile water;
5. Amplify for 40-50 cycles in a Light Cycler PCR machine and follow the precise manufacturer's instruction on how to

calculate the site-specific amount of DNA in the template DNA from which the real-time amplification was performed.

Duplex PCR Amplification

In a duplex PCR reaction a fragment from the region of interest and a control fragment (e.g. from the actin gene) are co-amplified. Primers should be designed in order to obtain comparable amplifications of the specific and control fragments when using a control genomic DNA as a template. It is also advisable to check that saturation of the amplification reaction (i.e. 30-35 cycles) will not change the ratio between the two PCR products. To work out precisely the ratio between the two different PCR products, it is best to perform the PCR reaction by adding radio-active dCTP (see protocol for PCR-SSCP: steps 1-8 of section titled PCR Amplification to Generate SSCP Polymorphisms). The radio-active PCR products should be run through a standard non-denaturing poly-acrylamide gel, as described in steps 16-21 of subsection on electrophoresis of digested PCR products. An example of a typical Duplex PCR assay, and its application to analyse immuno-precipitated chromatin fractions, is presented in Figure 3. (See note 11).

Allele Specific PCR Analysis of Precipitated Chromatin

Several PCR based methodologies exist to distinguish between alleles at loci of interest. If a polymorphic endonuclease restriction site is present in one allele and absent in the other, the method of choice is 'Hot-Stop' PCR. In case no polymorphic restriction sites are available, we feel that the best approach is to separate the PCR products derived from the two different alleles by using 'SSCP electrophoresis'.

Hot-Stop PCR Amplification Across a Polymorphic Restriction Site

Polymorphic restriction sites are used in many allele-specific PCR-based studies. During PCR amplification of DNA from mixed genetic background, hetero-duplexes (e.g. association of the opposite single strands) can be formed. Hence, the polymorphic restriction site will become non-digestible by the restriction enzyme and this will lead to too-high an estimation of the uncut material. Hot-Stop PCR is based on a standard cold amplification of the DNA, followed by addition of radio-labelled α^{32}-dCTP and fresh dNTPs for one single last cycle of 'hot' PCR amplification. Consequently, all radioactive products in the reaction are homo-duplexes at very low concentration, therefore, upon digestion by the restriction enzyme, the allelic ratio can be faithfully determined (Uejima *et al*., 2000).

For Hot-Stop PCR Amplification in a Final Volume of 25µl

1. Add 50-100ng of template DNA in a 0.2mL PCR tube;
2. Add forward and reverse primer to a final concentration of 0.4µM each;
3. Add 2.5µl of 10X buffer (supplied with the Taq polymerase);
4. Add dNTPs to a final concentration of 0.2µM;
5. Add water to a final volume of 25µl (remember the *Taq* polymerase);
6. Add 5 units of *Taq* polymerase (e.g. Hotstart-Taq enzyme, from Qiagen);
7. Amplify for 35-40 cycles in a thermal cycler;
8. Transfer 5µl of the PCR product to another PCR tube;
9. Complete to 25µl with a newly prepared PCR mix containing α^{32}-dCTP (10µCi, specific activity 3000Ci/mmol) and fresh dNTPs. Also add new *Taq* enzyme and oligonucleotide primers;
10. Amplify for one additional cycle only.

Restriction Enzyme Digestion of hot PCR Products

11. Transfer 10µl of the hot PCR product into a 1.5mL Eppendorf tube;
12. Add 1.5µl of 10x restriction enzyme buffer;
13. Add 10-20 units of the restriction enzyme specific for the polymorphic restriction site. Add sterile water to a final volume of 15µl;
14. Digest for 1-2 hours (for most enzymes, this will be at 37°C);
15. Add 5µl of Loading dye.

Electrophoresis of Digested PCR Products

16. Prepare the solution for the polyacrylamide gel: mix 15mL of acrylamide solution, 12mL of 5x TBE buffer and 32.5mL of de-ionized water. Add 50µl TEMED and 500µl freshly-prepared 10% APS;
17. Pour the gel immediately. Insert the shark-tooth comb, and clamp on all sides. Lay the gel flat, and let the matrix polymerise for at least 30 minutes;
18. After polymerisation, place the glass plates into the gel apparatus and add 1x TBE electrophoresis buffer;
19. Load samples into the gel and migrate at 120-200V for 2-3 hours;
20. Following gel electrophoresis, lay the gel on a sheet of Whatman 3MM paper and cover with plastic wrap. Dry for 45 minutes at 80°C in a gel dryer;
21. Expose the gel to an X-ray film at room temperature (for 4-16 hours). A phosphor-imager may be used to determine the

relative intensities of the bands.

PCR Amplification to Generate SSCP Polymorphisms

For studies on genomic imprinting, dosage compensation, and on other analyses of allelic gene expression, one needs to faithfully distinguish the parental origin of the alleles of a gene. If there are single nucleotide polymorphisms between the two alleles at the gene of interest, it is possible to discriminate (denatured) PCR products derived from the one or the other allele, because the secondary structure of each single-strand will be directly dependent on the sequence itself. Hence, in non-denaturing gel conditions each single strand will migrate differently (see figure 4). This technique is referred to as 'Single-Strand Conformation Polymorphism' (SSCP, Gregory *et al*, 2002) (see also note 12).

Radio-active PCR Amplification for SSCP Analysis

1. Add 50-100 ng of template DNA in a 0.2mL PCR tube;
2. Add forward and reverse primer to a final concentration of 0.4 M each;
3. Add 2.5µl of 10X buffer (supplied with the *Taq* polymerase);
4. Add dNTPs to a final concentration of 0.2µM;
5. Add water to a final volume of 25µl (remember the *Taq* polymerase);
6. Add 5 units of *Taq* polymerase (e.g., Hotstart-Taq enzyme, from Qiagen);
7. Add 1µl of α32-dCTP (10µCi/µl);
8. Amplify for 35-40 cycles in a thermal cycler.

SSCP Electrophoresis of Radio-active PCR Products

9. Prepare the solution for the non-denaturing MDE® gel (a poly-acrylamide-like matrix, specifically optimized for SSCP): mix 15mL of 2xMDE® solution, 7.2mL of 5x TBE buffer and 37.5mL of de-ionized water. Add 40µl TEMED and 400µl freshly-prepared 10% APS;
10. Pour the gel immediately. Insert the shark-tooth comb with teeth pointing upward to form a single well the width of the gel and clamp on all sides. Lay the gel flat, and let the matrix polymerise for at least 30 minutes;
11. After polymerisation, remove clamps, tape and comb. Place into the sequencing gel apparatus;
12. Take 2µl of PCR product and add 8µl of Loading dye. Denature the sample at 95°C for 5 minutes, then place on ice;
13. Load 5-7µl of the sample into the gel. Run the gel at 400V for 24 hours (at room temperature) (see note 12);
14. Following gel electrophoresis, lay the gel on a sheet of Whatman 3MM paper and cover with plastic wrap. Dry for 45 minutes at 80°C in a gel dryer;
15. Expose the gel to an X-ray film at room temperature (for 4-16 hours). A phosphor-imager may be used to determine the relative intensities of the bands.

Materials & Reagents

Buffer I	0.3M Sucrose in 60mM KCl 15mM NaCl 5mM $MgCl_2$ 0.1mM ethylene glycol-bis N,N,N′,N′-tetra-acetic acid (EGTA) 15mM Tris-HCl (pH 7.5) 0.5mM Dithiothreitol (DTT) 0.1mM phenylmethylsulfonyl fluoride (PMSF) 3.6ng/mL aprotinin (Sigma) See **note 1**, **note 2** and **note 7**.
Buffer II	0.3M sucrose in 60 mM KCl 15mM NaCl 5mM $MgCl_2$ 0.1mM EGTA 15mM Tris-HCl (pH 7.5) 0.5mM DTT 0.1mM PMSF 3.6ng/mL aprotinin 0.4% (v/v) IGEPAL CA-630® (formally called ‘Nonidet®P40’, from Sigma)
Buffer III	1.2M sucrose in 60 mM KCl 15mM NaCl 5mM $MgCl_2$ 0.1mM EGTA

	15 mM Tris-HCl (pH 7.5) 0.5mM DTT 0.1mM PMSF 3.6ng/mL aprotinin Parafilm® (Sigma)
MNase digestion buffer	0.32M sucrose 50mM Tris-HCl (pH 7.5) 4mM $MgCl_2$ 1mM $CaCl_2$ 0.1mM PMSF
tubing preparation solution I	2% (w/v) sodium bicarbonate 1 mM EDTA (pH 8.0)
tubing preparation solution II	1 mM EDTA (pH 8.0)
dialysis buffer	1mM Tris-HCl (pH 7.5) 0.2mM EDTA 0.2mM PMSF
loading buffer	6x concentration: 30% (v/v) glycerol in H_2O 0.25% (w/v) bromophenol blue 0.25% (w/v) xylene cyanol FF (store at 4°C).
1x TBE electrophoresis buffer	0.09M Tris-Borate 2mM EDTA (pH 8.0)
ChIP incubation buffer	50mM NaCl 50mM Tris-HCl (pH 7.5) 0.1mM PMSF 5mM EDTA.
Washing buffer A	50mM Tris-HCl (pH 7.5) 10mM EDTA 75mM NaCl Store at 4°C.
Washing buffer B	50mM Tris-HCl (pH 7.5) 10mM EDTA 125mM NaCl Store at 4°C.
Washing buffer C	50mM Tris-HCl (pH 7.5) 10mM EDTA 175mM NaCl Store at 4°C.
1X TE buffer	10mM Tris-HCl (pH 7.5) 1mM EDTA
5x TBE buffer	0.45M Tris-borate 10mM EDTA

Loading dye	95% (v/v) formamide 10mM NaOH 0.25% (w/v) bromophenol blue 0.25% (w/v) xylene cyanol
Elution buffer	50mM NaCl 50mM Tris-HCl (pH 7.5) 0.1mM PMSF 5mM EDTA 1% SDS (w/v)

Nuclei Preparation from Tissues and Cells

- Appropriate medium for culturing cells, e.g. RPMI containing 10% (v/v) foetal calf serum.
- Phosphate-buffered saline (PBS), pH 7.3.
- 0.05% (w/v) Trypsin solution (Sigma).
- Homogenizers: we use a tissue grinder/homogenizer (from BDH) that has a glass mortar (tube) and a pestle with a hard plastic head. The clearance between pestle and mortar is 0.15-0.25mm.
- 14mL polypropylene tubes (e.g. 17x100mm Falcon® tubes).
- Muslin cheese cloth.
- Buffer I
- Buffer II
- Buffer III
- Equipment: a high-speed centrifuge with a swing-out bucket rotor that takes 14mL polypropylene tubes.

MNase Fractionation and Purification of Chromatin

- MNase digestion buffer
- MNase (Amersham Bioscience), at 10units/µl in 50% (v/v) glycerol. 10-20µL aliquots are frozen and each aliquot should be used only once after thawing to ensure equal enzyme activity in different chromatin preparations.
- Water bath set at 37°C.
- Stop Solution: 20mM ethylene diamine tetra-acetic acid (EDTA), at pH 8.0.
- Bench-top centrifuge for 1.5mL Eppendorf tubes.
- Dialysis tubing, 0.5mm thick (VWR international).
- tubing preparation solution I
- tubing preparation solution II
- dialysis buffer
- 5mm universal tubing clamps (Spectrum-Laboratories).

Quality Control of Chromatin

- Spectrophotometer
- loading buffer, 6 times concentrated
- 10% (w/v) Sodium Dodecyl Sulphate (SDS)
- Horizontal gel electrophoresis tank for agarose gels
- 1x TBE electrophoresis buffer
- 100-base pair DNA size-ladder (Promega)

Chromatin Immuno-precipitation

- Protein-A (e.g., CL-4B sepharose from Amersham Bioscience), or G sepharose, according to the characteristics of the antibody used for immuno-precipitation (see note 9).
- ChIP incubation buffer
- Affinity-purified antiserum which were raised against histone peptides with mono, di, or tri-methylation at a specific lysine/arginine residue. We use ~5-10µg of antibody for ChIP on chromatin corresponding to ~20µg of genomic DNA.
- Bench-top centrifuge for 1.5mL Eppendorf tubes.
- Washing buffer A
- Washing buffer B
- Washing buffer C
- 15mL polypropylene tubes (e.g. 17x120mm Falcon® conical tubes).
- Centrifuge with a swing-out bucket rotor that takes 15mL polypropylene tubes.
- Elution buffer

DNA Extraction and Assessment of Precipitated Chromatin

- Phenol:chloroform:iso-amylalcohol 25:24:1 (v/v/v). For extraction of genomic DNA, the phenol should be saturated beforehand with 100 mM Tris-HCl (pH 7.5), and stored at 4°C under 10mM Tris-HCl (pH 7.5) (27).
- 5M NaCl
- Glycogen solution at 20mg/mL (Roche)
- Iso-propanol
- 70% (v/v) ethanol
- 1X TE buffer
- Spectrophotometer
- loading buffer, 6 times concentrated
- 10% (v/v) SDS
- Horizontal electrophoresis tank for agarose gels
- 1x TBE electrophoresis buffer
- 100-base pair DNA size-ladder (Promega)
- Ethidium-Bromide solution, at 20 mg/ml in H_2O

Quantitative PCR analysis of Precipitated Chromatin

- Template DNA: the genomic DNA extracted from the antibody-bound and antibody-unbound fractions. Control genomic DNAs should be used as well. For each PCR amplification we use 20-50ng of template DNA.
- Quantitect SYBR Green PCR kit (QIAGEN).
- LightCycler machine (e.g. Roche Diagnostics).

Allele-Specific PCR Analysis of Precipitated Chromatin

Hot-Stop Amplification Across Polymorphic Restriction Sites

- Template DNA (50-100ng of DNA)
- Forward and reverse primers (stock solutions in H_2O at 100µM)
- dNTPs mix (stock solutions at 25mM for each dNTP)
- α^{32}P-dCTP (10µCi/µl, specific activity 3000 Ci/mmol)
- 10X PCR amplification buffer (supplied with the Taq polymerase)
- Taq polymerase (at 5U/µl)
- Thermal cycler
- 0.2-mL thin-walled PCR tubes (e.g. from EUROGENTEC)
- Restriction endonuclease specific for a polymorphic restriction site within the amplified DNA fragment
- 10X digestion buffer (supplied with the restriction endonuclease)

Electrophoresis of Restriction Enzyme-digested PCR Products

- Acrylamide/bis-acrylamide 40% stock solution (29:1 ratio) (Sigma)
- 1x TBE eletrophorsis buffer
- 5x TBE buffer
- N,N,N',N'-tetramethyl-ethylene diamine (TEMED)
- 10% (w/v) ammonium persulphate (APS), freshly prepared
- Vertical gel electrophoresis tank for poly-acrylamide gels with 21.7x16.5cm glass plates, 0.4mm spacers and a shark-tooth comb
- PCR product
- loading buffer, 6 times concentrated
- Whatman® 3MM paper
- Thin transparent plastic wrap (e.g. Saran® Wrap)
- Gel dryer for acrylamide gels (e.g. Biorad® model 583)

SSCP-PCR Amplification and electrophoresis of amplification products

- Acrylamide/bis-acrylamide 40% stock solution (29:1 ratio) (Sigma)
- 1X TBE buffer
- N,N,N',N'-tetramethyl-ethylene diamine (TEMED)
- 10% (w/v) ammonium persulphate (APS), freshly prepared
- Vertical gel electrophoresis tank for poly-acrylamide gels with 21.7x16.5cm glass plates, 0.4mm spacers and a shark-tooth comb
- PCR product
- loading buffer, 6 times concentrated
- Whatman® 3MM paper
- Acrylamide solution for SSCP gels: 2xMDE® solution (Sigma)

- 5x TBE buffer
- N,N,N',N'-tetramethyl-ethylenediamine (TEMED)
- 10% (w/v) ammonium persulphate (APS), freshly prepared
- A standard DNA sequencing gel apparatus with 31x38.5cm glass plates, 0.4mm spacers and a shark tooth comb
- PCR product
- Loading dye
- Whatman® 3MM paper
- Thin transparent plastic wrap (e.g. Saran® Wrap)
- Gel dryer for acrylamide gels (e.g. Biorad® model 583)

Gel Image Analysis

- X-ray films. Cassettes with scintillation screens for exposure of X-ray films
- Imaging equipment for densitometric measurements on exposed X-ray films (e.g. Geldoc-1000 system from Biorad)
- Phosphor-imager (e.g., Molecular Imager FX system from Bio-Rad)

Authors Notes

1. Wear gloves throughout all procedures and respect the other safety rules, especially when handling phenol, chloroform and acrylamide solutions.
2. The described protocols work well on tissues, such as liver, brain, lung and placenta, and also on early embryos. In our laboratory, for instance, we have performed studies on 8.5-9.5 d.p.c. mouse embryos and placentas (Umlauf *et al*., 2004). For these experiments we used some 40-80 dissected embryos for each ChIP experiment. No more than ~0.2 grams of tissue should be used for the volumes and tube-sizes indicated in the protocol. In case higher amounts of tissue are needed, we recommend to increase the number of tubes accordingly. To analyse histone acetylation in the same sample used for assaying histone methylation, we recommend to add sodium butyrate (to a final concentration of 5 mM) to the solutions used for the purification of nuclei and the preparation of input chromatin. Sodium butyrate prevents loss of histone acetylation via the action of endogenous histone de-acetylases. For the site-specific analysis of histone phosphorylation, different protocols are applied, which are described elsewhere (Clayton *et al*., 2000; Thomson *et al*., 2001), with addition of specific inhibitors of phosphatases.
3. For many tissues (liver, kidney, placenta), a 0.2 % concentration of the non-ionic detergent IGEPAL CA-630 will be sufficiently high to lyse the cellular membranes during the 10-minutes incubation. However, we recommend testing 0.4% IGEPAL for certain other tissues. For example, this higher concentration of detergent slightly improves the yield of nuclei from brain and muscle tissues.
4. At step 4 of this procedure, it is critical not to extend the incubation in the IGEPAL CA-630®-containing buffer for more than 10 minutes: start the subsequent centrifugation step at exactly 10 minutes after adding Buffer II to the cells re-suspended in Buffer I.
5. At step 6 of the nuclei purification procedure, the nuclei pellet should be white. For liver nuclei preparation, for instance, all the red (due to the presence of haemoglobin in the blood cells) should be in the layer on top of the sucrose cushion. At step 7, no trace of the top layer (containing the IGEPAL CA-630®) should come into contact with the nuclei pellet. Usually, we remove the top layer and the sucrose cushion from the tube by using Pasteur pipettes. This is done by aspirating from the surface of the solution, while changing very often the Pasteur pipette. If the top layer had nevertheless come in contact with the nuclei pellet, the pellet should be gently rinsed with 1 mL of Buffer III once, before proceeding with step 8.
6. Grind the frozen tissue to powder in a mortar, while keeping it constantly under liquid nitrogen (pre-chill the mortar with liquid nitrogen). Transfer the finely ground tissue into 5-10 mL of ice-cold Buffer I, and re-suspend the material. Filter this suspension through two layers of muslin cheese cloth that has been moistened beforehand with 2 mL of Buffer I. Proceed from here with step 3 of the nuclei purification procedure.
7. Protease inhibitors, and in particular PMSF, are rather unstable in aqueous solutions. Therefore, PMSF (and the DTT and aprotinin) should be added to the relevant solutions (Buffers I-III, and solutions used thereafter) as late as possible, just before they are being used (see comment 1).
8. Chromatin immuno-precipitations and incubations with Protein-A (G) Sepharose are performed in Eppendorf tubes. These tubes may be siliconised beforehand (e.g., with a 2% v/v dichloromethylsilane solution), in order to prevent aspecific association of chromatin and antibodies to the inner wall of the tubes. In our laboratory (Goto *et al*., 2003; Fournier *et al*., 2002), however, we obtained comparable results with non-siliconised and siliconised tubes.
9. Protein-A and G are bacterial cell wall proteins that bind to the Fc region of antibodies. This interaction is strongest at neutral, or slightly basic pH values. These proteins are covalently coupled to Sepharose. The choice between protein-A or protein-G Sepharose depends on the nature of the antibody used for ChIP. In general, Protein-A works best for rabbit polyclonal antisera and for mouse monoclonal antibodies from the IgG2a,b and IgG3 subclasses. Protein-G Sepharose should be preferred for mouse IgG1 mono-clonal antibodies, and for polyclonal antisera from mouse, rat, sheep and goat. Chicken antisera do not bind well either to protein-A or protein-G, therefore, when used for ChIP, we recommend adding 5 µg of a rabbit anti-chicken antiserum directly after step 4 of chromatin immuno-precipitation, for a second precipitation of 3-4 hours, before proceeding with the extraction of the antibody-bound chromatin.
10. The percentage of input chromatin that is precipitated is not the same for different histone modifications. Factors which can influence the outcome of the experiment are: 1) the distribution of the histone modification on the chromosomes; 2)

the amount of antiserum used, and 3) the 'strength' of the antibodies (i.e., the affinity for their epitope). On the other hand, the efficiency of precipitation of modified histones at a locus of interest greatly depends on whether the modification is common, or rare in the genome. For instance, a modification that is rare in the genome (e.g., H3-K4 methylation, see Figure 4) gives usually good precipitation at the site at which it is present. This can be explained by the fact that in the ChIP the quantity of antibody added to the tube is high enough to precipitate all the chromatin that carries that specific modification. However, for a modification which is abundant in the genome, the indicated amount of antibody (5-10 µg) does sometimes not permit to precipitate all the chromatin that has the modification.

11. Analysis of precipitated chromatin fractions by PCR requires extreme care. Depending on the amount of input chromatin and the abundance of the modification at the site of interest, sometimes only little DNA template will be available for amplification. We recommend taking all possible precautions (Sambrook & Russell, 2001) to prevent contamination from other DNA sources: amplification in a dedicated space (PCR hood), use of a set of pipettes for PCR only, use of filter tips, etc.
12. In most cases SSCP separates 150-300 bp single-stranded DNA fragments with one, or more, nucleotide difference (Orita *et al*., 1989). However, the migration of single- stranded fragments in the gel is strongly temperature-dependent. Ideally, therefore, the PCR samples to be compared should be run on the same gel. In addition, SSCP is more efficient for DNA with a relatively high G+C content. SSCP analysis of fragments with a lower G+C content can be enhanced by electrophoresis at 4°C. Instead of adding radioactive α^{32}P-dCTP to the PCR reactions for SSCP analysis, the PCR primers (forward and reverse) may be radioactively end labelled by using T4 polynucleotide kinase and α^{32}P-dATP.

Reviewer Comments

Reviewed by: Annabelle Lewis, The Babraham Institute, Cambridge, UK.

1. I have used the protocol successfully. There are a couple of things one should draw more attention to. The first is that some protease inhibitors, in particular, PMSF are unstable in aqueous solutions so they should be added to buffers (I, II, III, digestion, dialysis and ChIP incubation) immediately before use. I also can't remember reading that sucrose containing buffers should also not be kept for too long.

   They should also state more clearly that when making up the ChIP incubation buffer the volume and concentrations of NaCl, Tris-HCl and EDTA present in the S1 (in digestion buffer) and S2 (in dialysis buffer) fractions should be taken into account.

Figures

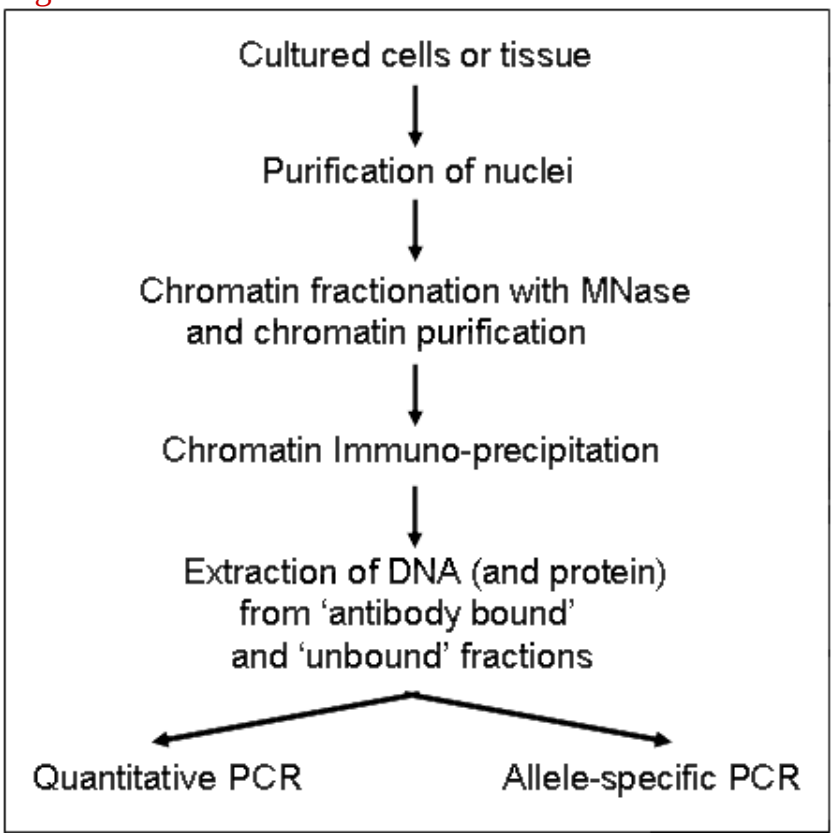

Figure 1.
Schematic of the protocol used to investigate site-specific covalent modifications (i.e. methylation and acetylation) on histones. In summary, nuclei are purified from fresh/frozen tissues, or from cells, and the chromatin, after fractionation with micrococcal nuclease, is purified from the nuclei. This 'input chromatin', made up of fragments of up to five nucleosomes in length, is incubated with an antiserum directed against the histone modification of interest. Subsequently, the antibody-bound fraction is

separated from the unbound fraction, genomic DNA is extracted from the bound and unbound fractions, and PCR technologies are applied to specifically analyse the gene or chromosomal region of interest.

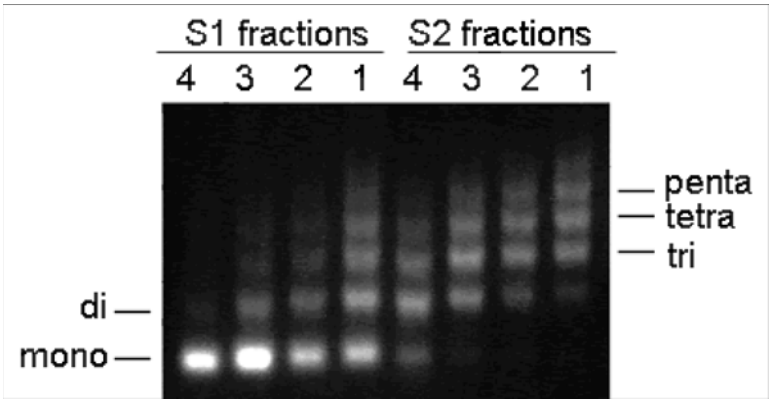

Figure 2.
Photograph of native chromatin preparation with fragments of on average one to five nucleosomes in length. For this experiment, nuclei were purified from primary fibroblast cells and incubated with MNase for 6, 9, 12 and 15 minutes (lanes 1-4, respectively). The S1 fractions were obtained directly after MNase digestion, whereas the S2 fractions were recovered by overnight dialysis. Bands corresponding to chromatin fragments of one nucleosome (mono) to five nucleosomes (penta) in length are indicated (1.2% agarose gel). Fractions 1 and 2 of S1 were combined with fractions 3 and 4 of S2 for subsequent ChIP (adapted from Goto *et al*. 2003).

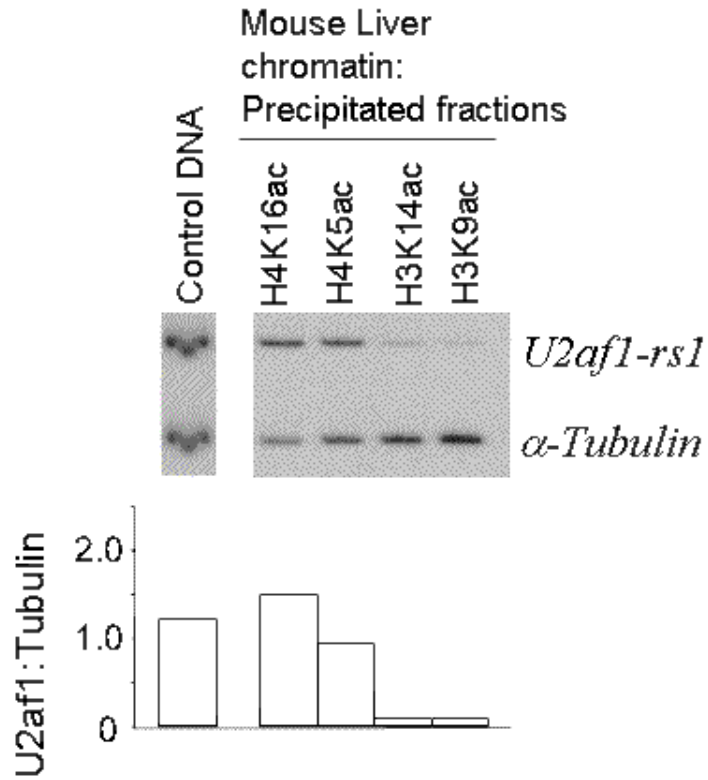

Figure 3.
Quantitative Duplex PCR amplification of precipitated chromatin. Gregory and co-workers (2001) used "Duplex PCR" to investigate histone modifications at the splice-factor-encoding U2af1-rs1 gene (site of interest) which were compared to those at the Tubulin gene (control locus). Two sets of PCR primers, one for the splice-factor-encoding gene U2af1-rs1 and one for the Tubulin gene, were used in a single PCR reaction ("Duplex PCR") to check that they amplified similar amounts of DNA from a control genomic DNA (left lane in the Figure). Chromatin fractions (see Figure 2 and Subheadings titled Nuclei Preparation from Tissues and Cells and Fractionation and Purification of Chromatin), from native liver chromatin, were then immunoprecipitated with antisera directed against acetylation at lysine residues 16 and 5 on histone H4, and against acetylation at lysines 14 and 9/18 of histone H3, respectively (for description of these antisera, see O'Neill & Turner, 1995; Gregory *et al*., 2001). After duplex amplification from each of the antibody-bound fractions, and electrophoresis through a gel, the intensity of the U2af1-rs1-specific PCR product was compared to that of the Tubulin product. The calculated U2af1-rs1:Tubulin ratios are plotted underneath. (Figure adapted from Gregory *et al*. 2001).

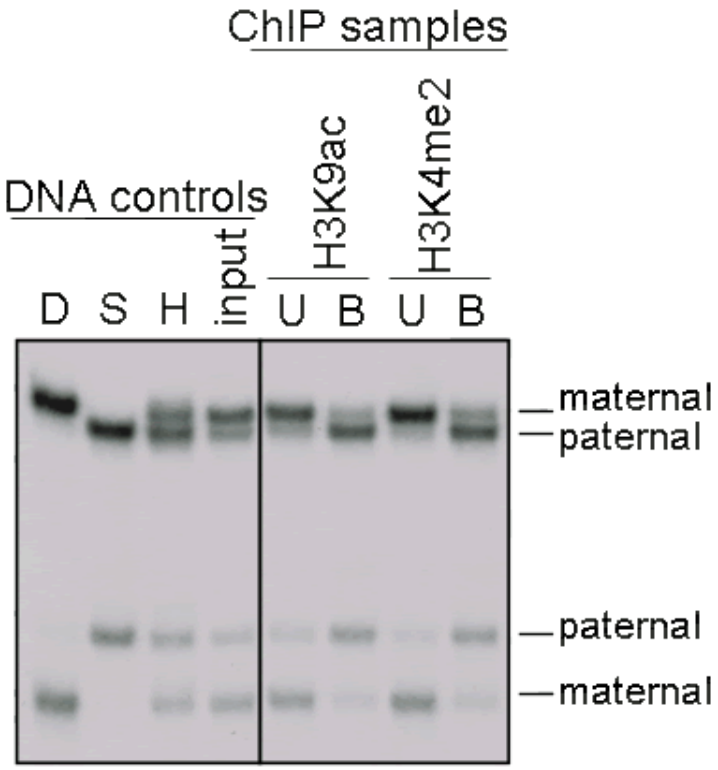

Figure 4.
Allele-specific patterns of histone modification revealed by PCR amplification and SSCP electrophoresis. Lung tissue was dissected from a mouse which was an inter-specific hybrid (H) between Mus musculus domesticus (D, paternal genome) and Mus spretus (S, maternal genome). The native chromatin was then immuno-precipitated with rabbit polyclonal antisera to acetylation at lysine 9 of H3 (H3K9Ac) and to di-methylation at lysine 4 of H3 (H3K4Me2) (Upstate Ltd. and Fournier *et al*., 2002). Radio-active PCR was performed on bound (B) and unbound (U) fractions with a pair of primers that amplified from a unique sequence at an imprinting-control centre which is located in a gene called Kvlqt, and which controls the parental allele-specific expression of a cluster of imprinted genes on distal mouse chromosome 7. The PCR products were denatured and subjected to electrophoresis through a non-denaturing poly-acrylamide gel (SSCP electrophoresis). The four lanes to the left show control amplifications from genomic DNAs (D, *Mus musculus domesticus* DNA; S, *Mus spretus* DNA; H, (*Mus musculus domesticus* x *Mus spretus*) F1 DNA. In the analysis of the antibody bound (B) and unbound (U) fractions (right panel), the bands representing the maternal and paternal alleles are indicated.

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