Analysis of chromatin in limited numbers of cells: a PCR–SSCP based assay of allele-specific nuclease sensitivity

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ABSTRACT

Chromatin can be analysed by assaying its sensitivity to DNase I or other nucleases in purified nuclei. Usually, this is performed by Southern analysis of genomic DNA extracted from nuclease-treated nuclei, a methodology that requires many cells. Applying restriction fragment length polymorphisms (RFLPs), this methodology has been used for parental allele-specific chromatin studies on imprinted mammalian genes. However, such allelic studies are limited by the availability of suitable RFLPs. We therefore developed an alternative, PCR and single strand conformation polymorphism (SSCP)-based assay with which allelic sensitivity to nucleases can be determined in virtually all localised regions that have nucleotide polymorphisms. We also demonstrate that analysis of DNase I sensitivity can be performed on permeabilised cells. Combining the two approaches, in the imprinted mouse *U2af1-rs1* gene we analysed parental allele-specific chromatin conformation in limited numbers of cultured cells. We also applied the PCR–SSCP approach to assay allelic DNA methylation at specific restriction enzyme sites. In summary, we developed an allele-specific assay that should be useful for biochemical and developmental investigation of chromatin, in particular for studies on genomic imprinting and X-chromosome inactivation.

INTRODUCTION

The conformation of chromatin can be studied by determining its sensitivity to DNase I, or other endonucleases, in specific chromosomal regions. In the most commonly used methodology, purified nuclei are incubated at increasing concentrations of DNase I after which genomic DNA is extracted and analysed by Southern blot hybridisation (1). For many eukaryotic genes, it has thus been established that their chromatin is more sensitive when they are in a (potentially) active state than when they are transcriptionally repressed (2). More recently, by using restriction fragment length polymorphisms (RFLPs) in the Southern analysis, a modification of this methodology has allowed analysis of (imprinted) genes for their allele-specific chromatin conformation (3,4). Distinct chromatin differences between the parental alleles have thus been demonstrated in the imprinted *H19*, *U2af1-rs1* and *SNRPN* genes in the mouse (5–7). However, suitable RFLPs are frequently not available in genomic regions of interest and it is therefore important to develop an alternative strategy to discriminate the parental alleles. In addition, to be able to perform developmental studies on early embryos and germ cells, one requires a methodology with which small numbers of cells can be analysed (the Southern-based method necessitates at least 5×10^7 cells; 1,4).

Here we describe a PCR-based assay of DNase I sensitivity that does not involve purification of nuclei and therefore allows studies on limited numbers of cells. In addition, to distinguish the maternal and paternal chromosomes in localised regions of interest, and to carefully assess their relative sensitivity to nucleases, it involves the use of single strand conformation polymorphism (SSCP), a method widely used for mutation detection (8,9).

To test the usefulness of the proposed PCR–SSCP approach (outlined in Table 1), we first demonstrated that it faithfully assays the relative sensitivity of the parental chromosomes in the mouse *U2af1-rs1* gene. Previously, we have shown that this (paternally expressed) imprinted gene and its direct flanking sequences have a higher 'generalised sensitivity' to DNase I on the paternal than on the maternal chromosome in all tissues analysed (5). For the current study, we purified nuclei from (C57Bl/6 × *Mus spretus*) F1 kidney and these were incubated at increasing concentrations of DNase I, as described in detail before (4,5). After extraction of genomic DNA, Southern blot hybridisation was performed using a *Bgl*II RFLP between C57Bl/6 and *M.spretus* (Fig. 1A). Hybridisation of the blot with a radioactively labelled probe from the *U2af1-rs1* gene demonstrated that the paternal allele was significantly more sensitive than the maternal allele. Subsequently, the DNA samples extracted from the kidney DNase I series were used as templates for PCR amplification with primers from the 5′-portion of the *U2af1-rs1* gene (see below). The selected pair of primers (U2f and U2r) amplifies a 293 bp fragment that has several base differences between C57BL/6 and *M.spretus*. Because of these sequence polymorphisms, the C57BL/6- and *M.spretus*-specific PCR products can be readily distinguished by SSCP. Hence, after denaturing of the PCR products to single-stranded molecules, these fragments migrate at different positions in a non-denaturing polyacrylamide gel (Fig. 1B). Of critical importance relative to the proposed qualitative use of

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Table 1. Assay of allelic DNase I sensitivity in cells and pre-implantation embryos

1.	Cells: collect cultured cells (>5000), by trypsinisation followed by centrifugation in PBS. Preimplantation embryos: embryos (>50) of the desired genotype and developmental stage, produced by standard mouse embryology techniques.
2.	Re-suspend cells or embryos in 30 µl of ice-cold DNase I buffer: 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 15 mM Tris–HCl (pH 7.5) Aliquot 5 µl into each of six, numbered, 0.5 ml Eppendorf tubes. Add 5 µl of ice-cold DNase I buffer containing 0.4% Nonidet™ P40 to each of the tubes, mix gently, and incubate on ice for 5 min. (Note: do not extend incubation for longer.)
3.	Add 10 µl of DNase I buffer containing 1 U/µl DNase I (Boehringer Mannheim, grade I) and incubate at 25°C. (Note: the precise amounts of DNase I need to be optimised for each region.)
4.	At various time points (e.g. at 0, 0.5, 1, 2, 3 and 5 min after addition of DNase I), heat inactivate the DNase I by incubation at 95°C for 20 min. After heat inactivation of the whole series, add proteinase K to a concentration of 200 µg/ml, and incubate at 50°C overnight.
5.	Heat inactivate the proteinase K by incubation at 95°C for 1 h.
6.	PCR amplification. Take 2–5 µl per sample and amplify (30–40 cycles) in a total volume of 30 µl of PCR buffer with each dNTP at 100 µM, 1 µCi [^{32}P]dCTP, 1.5 U *Taq* polymerase. As an alternative method of radiolabelling the PCR products, end-labelled primers can be used instead of adding [^{32}P]dCTP to the reaction (8,9). (Note: amplification conditions should be optimised for each selected primer pair; 10.)
7.	SSCP analysis. Take 1 µl of the PCR product and add to 10 µl of loading dye (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol). Denature at 95°C for 3 min and place on ice. Then take 1–3 µl and migrate (at room temperature, for 16–24 h at 4–8 W, in 0.6× TBE buffer) on a 0.4 mm thick non-denaturing polyacrylamide gel (MDE™ gel solution; FMC), using a 36 cm gel tank.
8.	After migration, the gel is transferred onto Whatman® 3MM paper, vacuum dried in a gel drier for 45 min and exposed overnight to an X-ray film. For precise quantitation of relative band intensities it is best to expose the gel to a phosphorimager.

SSCP, in the U2f-U2r PCR product amplified from (C57BL/6 × *M.spretus*) F1 kidney DNA, the C57BL/6- and *M.spretus*-specific single-stranded fragments were of the same intensities. Comparable results were obtained for PCR fragments amplified from elsewhere in the *U2af1-rs1* locus (data not shown). SSCP analysis presents an improvement as compared to the commonly used approach of endonuclease digestion to distinguish allele-specific PCR products. To exemplify the difference between the two approaches, we digested the same U2f/U2r PCR products with *Bgl*I, a restriction enzyme which has a recognition site only in the C57BL/6-specific U2f/U2r PCR product. The PCR product amplified from (C57BL/6 × *M.spretus*) F1 genomic DNA did not yield the expected 50% contribution of the C57BL/6-specific *Bgl*I digestion product (of 211 bp). In fact, a relative C57BL/6 contribution of only ~25% was detected (Fig. 1D). The strong bias against the C57BL/6 allele is most likely caused by heteroduplex formation (which prevents *Bgl*I digestion), a problem where PCR amplifications are sub-optimal or have become saturated (10). It has been shown, however, that for some primer pairs, heteroduplex formation can be minimised by a one-cycle, second round of PCR amplification (11).

Applying SSCP, we analysed (radiolabelled) PCR products amplified from the DNA samples of the DNase I series on (C57BL/6 × *M.spretus*) F1 kidney (Fig. 1B). This demonstrated that at increasing concentrations of DNase I, the paternal allele was significantly more sensitive than the maternal allele, a result comparable to that obtained in the Southern blot analysis of the same DNA samples (Fig. 1E).

We established that the PCR–SSCP approach can also be used to determine allele-specific levels of DNA methylation at specific CpG dinucleotides and this should be useful where suitable RFLPs are not detectable. This we demonstrated for three *Hpa*II restriction sites (Fig. 1C), and for a single *Sna*BI restriction site (not shown), that are within the U2f/U2r fragment. These, and all other recognition sites of methylation-sensitive restriction enzymes that we analysed in the *U2af1-rs1* gene, are methylated on the maternal chromosome exclusively (5). Hence, (C57BL/6 × *M.spretus*) F1 kidney DNA was digested with either *Hpa*II or *Sna*BI, after which PCR amplifications were performed with primers U2f and U2r. PCR products were denatured and analysed on an SSCP gel as described in Table 1. Only bands corresponding to the C57BL/6-specific fragments were visible, demonstrating that the *Hpa*II (and *Sna*BI; not shown) sites are methylated on the maternal (C57BL/6) but not on the paternal (*M.spretus*) chromosome (Fig. 1C).

With a qualitative PCR–SSCP assay established, we were interested in determining next whether allelic DNase I sensitivity can be assayed in small numbers of cells. We made use of the demonstration by Stewart *et al.* (12) that DNase I remains active in the presence of non-ionic detergents. Primary embryonic fibroblasts (congenic cell line EF1), derived from day 14 embryos, were analysed (as described in Table 1). Cells were incubated in a buffer containing 0.2% Nonidet™ P40 (5 min at 0°C), a mild treatment that permeabilises the cell wall but seems not to affect chromatin conformation (4,12). Incubations with DNase I were performed for increasing periods of time (for 0, 0.5, 1, 2, 3 and 5 min), and were arrested by heat inactivation at 95°C and proteinase K digestion at 50°C (see Table 1). PCR amplifications were performed with primers U2f and U2r, and amplification products were analysed on an SSCP gel. This showed a significantly higher DNase I sensitivity on the paternal than on the maternal chromosome, in two separate experiments on 1×10^4 cells (Fig. 2) and 5×10^3 cells (data not shown). This result was comparable to that obtained by Southern analysis of the same cell line (data not shown). In fact, performance of the DNase I digestions on permeabilised cells might give better results in some instances, since conventional nuclei purification methods can alter certain chromatin structures (13).

Finally, we applied the PCR–SSCP approach to the analysis of chromatin in pre-implantation mouse embryos. Mouse blastocysts (52×) that were of the (C57BL/6 × *M.spretus*) F1 genotype

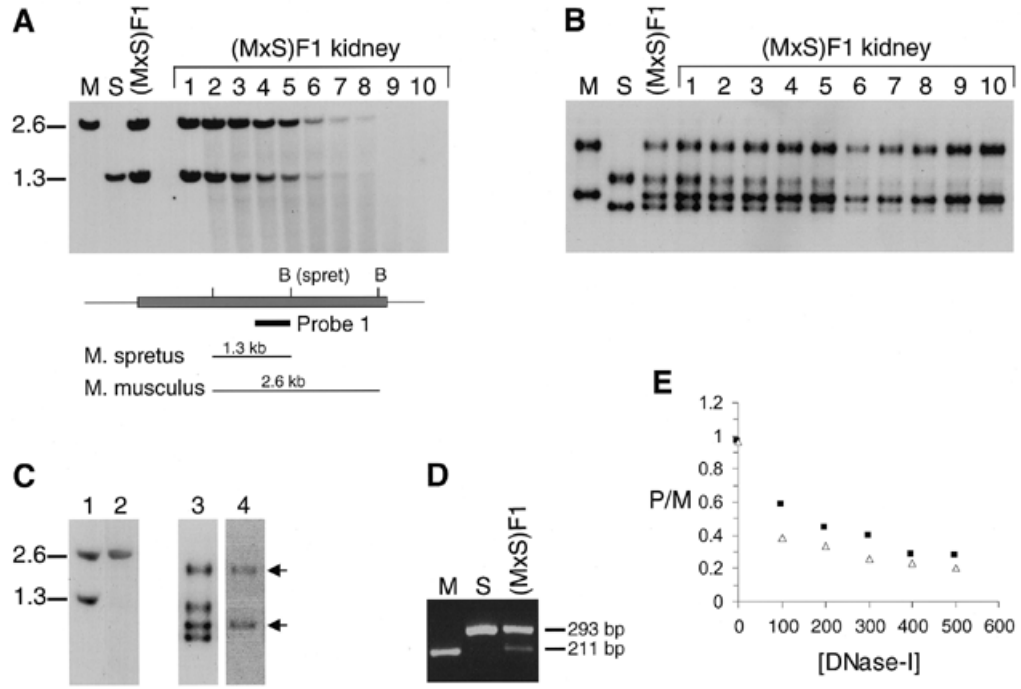

Figure 1. Analysis of parental allele-specific DNase I sensitivity in the *U2af1-rs1* gene. (**A**) The Southern-based methodology. Nuclei were purified from kidney tissue (4) dissected from three congenic mice that were (*M.musculus* × *M.spretus*) F1 for proximal chromosome 11 (to which *U2af1-rs1* maps). As described before (4), nuclei were incubated (for 10 min at 25°C) at increasing concentrations of DNase I (lanes 1–10 correspond to 0, 50, 100, 150, 200, 250, 300, 375, 500 and 750 U/ml). Genomic DNA samples were then extracted, re-digested with *Bgl*II + *Sac*I, migrated on a 1% agarose gel, transferred onto nylon membrane and hybridised with *U2af1-rs1* probe 1 (5). The map shows the *Bgl*II (B) and *Sac*I (Sa) restriction sites within the gene (shaded box), and the position of probe 1. In *Sac*I + *Bgl*II-digested *M.musculus* (M) DNA, probe 1 hybridises to a 2.6 kb fragment, whereas in *M.spretus* (S), a 1.3 kb fragment is detected. (**B**) PCR–SSCP-based analysis of the same kidney DNase I series (lanes 1–10). Primers U2f (CGCAGATCAGACATACTGCGG) and U2r (TGTGGTACGGCCAGCCTATG) were used to amplify a 293 bp fragment from the 5′-portion of the *U2af1-rs1* gene (GenBank accession no. D26474, nt 906–1199). Aliquots of 100 ng of genomic DNA were used as template, in a total volume of 30 µl containing 2 µM each primer, 200 µM each dNTP, 1 µCi [^{32}P]dCTP and 1.5 U *Taq* DNA polymerase. To denature template DNA, an initial step of 94°C (3 min) was chosen, followed by 33 cycles of 94°C (1 min), 60°C (1 min) 72°C (1 min) and a final step of 72°C (10 min). Denatured PCR products were run for 24 h at 4 W on a SSCP gel prepared according to the manufacturer's (MDE™ gel; FMC) instructions. After gel drying, band intensities were determined using a phosphorimager, and the sum of the two paternal single-stranded fragments was compared with the sum of the two maternal single-stranded fragments. (**C**) Southern-based methylation analysis of genomic kidney DNA digested with *Bgl*II (lane 1) or *Bgl*II + *Hpa*II (lane 2), and hybridised with probe 1. The same enzyme-digested DNA samples were analysed by PCR–SSCP as described in (B) (lane 3, *Bgl*II-digested DNA; lane 4, *Bgl*II + *Hpa*II-digested DNA). Arrows indicate the maternal chromosome-specific single-stranded fragments. (**D**) RFLP digestion of PCR products was carried out on PCR products [the same as in (A)] amplified from the *M.musculus* (M), *M.spretus* (S) and (*M.musculus* × *M.spretus*) F1 genomic DNA. *Bgl*I-digested PCR products were run on a 1.2% agarose gel and stained with ethidium bromide. (**E**) Relative DNase I sensitivity of the parental alleles in *U2af1-rs1* assayed by Southern blotting (triangles) and by PCR–SSCP (squares). The ratios between paternal and maternal band intensities shown in (A) and (B) are presented.

for proximal chromosome 11 (where *U2af1-rs1* resides), were incubated with the Nonidet™ P40-containing buffer after which DNase I digestions were performed for increasing periods of time, in the same way as for the fibroblasts. Upon PCR amplification and SSCP analysis, the paternal allele was found to be more sensitive to DNase I digestion than the maternal allele (data not shown).

From our data it follows that SSCP analysis of PCR products provides a faithful representation of the parental alleles in assays of nuclease sensitivity. Unlike other allele-specific assays of chromatin and DNA methylation, our PCR–SSCP assay is based on conformational characteristics of small single-stranded fragments (8,9). Results are therefore not influenced by the extent to which amplifications become saturated, a recurrent problem that affects qualitative analysis by restriction endonuclease digestion of PCR products (10,11). A second advantage of the PCR–SSCP approach is that in almost all regions of interest it should allow one to tell the parental chromosomes apart in cells with genetically different maternal and paternal genomes; given the scarcity of RFLPs, this would be particularly beneficial for allelic studies on human genes. Single-stranded PCR fragments are readily separated by non-denaturing gel electrophoresis and in 80–100% of cases, a single base change is sufficient to cause altered electrophoretic mobility there were short PCR products (150–300 bp) are analysed (8,9,14). Finally, the proposed PCR–SSCP approach is technically not complicated and requires only one round of PCR amplification. In this study we also established that the PCR–SSCP approach might be applied to analyse small numbers of permeabilised cells. This scaling down (from $>5 \times 10^7$ to

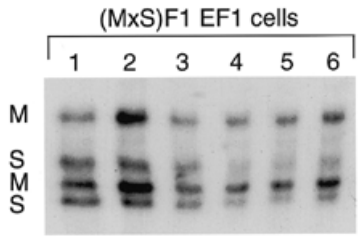

Figure 2. PCR–SSCP-based analysis of nuclease sensitivity in limited numbers of cells. Early passage, primary embryonic fibroblasts [that were (*M.musculus* × *M.spretus*) F1 for proximal chromosome 11] were trypsinised. Samples of 1×10^4 cells were distributed equally among six tubes. DNase I digestion was carried out as described in Table 1, using 500 U/ml DNase I. Reactions were stopped by heat inactivation, after 0, 0.5, 1, 2, 3 and 5 min of incubation for tubes 1–6 (lanes 1–6, respectively). PCR amplifications (with 3 µl of the sample, 38 cycles) and SSCP analysis were performed as described in Figure 1B.

5×10^3 cells) should allow chromatin conformational studies on pre-implantation embryos and germ cells. Although we limited our study to the endonuclease DNase I, the described approach should work for other endonucleases (micrococcal nuclease and restriction endonucleases) as well, with appropriate modification of the digestion buffer (5,12). In addition, it could also be useful for other chromatin investigations that necessitate distinction of parental chromosomes. For example, we are currently applying the PCR–SSCP approach to study allele-specific levels of core histone acetylation in the imprinted *U2af1-rs1* domain (R.I.Gregory, unpublished results). Another application of PCR–SSCP that we established in this study is the analysis of allele-specific DNA methylation at specific restriction sites. We show for *U2af1-rs1* that the PCR–SSCP approach gives a good representation of the allelic levels of methylation. Methylation studies involve, however, digestion of purified (double-stranded) DNA with (methylation-sensitive) restriction endonucleases and can therefore not be readily performed on limited numbers of cells. Finally, although this is a qualitative assay, it might be used for non-allelic, quantitative studies of chromatin as well, by including appropriate control amplifications on the same DNase I-treated samples (15). In summary, we describe a technically simple, qualitative assay of nuclease sensitivity with which chromatin studies can be performed on limited numbers of cells. This novel approach should therefore be useful for developmental and biochemical investigations of eukaryotic chromatin, in particular for studies on imprinted and X-linked mammalian genes.

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