Analysis of Chromatin Structure by Ligation-mediated PCR

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The expression of eukaryotic genes is mediated at the transcriptional level by a complex interplay between *trans*-acting factors and *cis*-acting elements. Interactions of proteins with specific DNA sequences are most commonly studied in vitro by applying the popular gel mobility-shift assays and footprinting techniques. These experiments are usually done using nuclear extracts from cell lines or tissues of interest. Beyond this type of analysis, it is desirable to have evidence that a protein which complexes with a particular DNA sequence in the test tube is actually bound to the same sequences under in vivo conditions. Within the cell, the accessibility of a sequence to a *trans*-acting factor may be restricted by a particular chromatin environment. Dynamic interactions of protein factors with their DNA target may be accompanied by changes in chromatin structure. These complex situations and processes are difficult to reproduce in vitro.

To investigate protein–DNA contacts in living cells, in vivo footprinting strategies have been devised.^(1,2) These methods are based on direct genomic sequencing⁽³⁾ where total genomic DNA is subjected to base-specific chemical cleavage and the sequence of a particular single-copy gene is then revealed without conventional cloning procedures. For complex genomes, such as those of mammals, special techniques are required to accomplish this goal. The most sensitive method uses the ligation-mediated polymerase chain reaction (LM-PCR).^(4–6) This method significantly increases the sensitivity and specificity of the original genomic sequencing methods. All of the component fragments of a DNA sequence ladder are simultaneously amplified by PCR and displayed as sequencing gel autoradiograms. LMPCR

preserves the quantitative representation of each fragment of the sequence ladder in the original population of cleaved molecules and each sequence ladder can be amplified very reproducibly. This has made possible its use for in vivo footprinting,⁽⁴⁾ genomic sequencing,⁽⁵⁾ methylation analysis,^(5,7,8) and studies on chromatin structure at single-nucleotide resolution.⁽⁹⁾

LIGATION-MEDIATED PCR

The starting material for LMPCR is total genomic DNA that has undergone a specific cleavage procedure to result in a DNA strand break or nick. Either chemical or enzymatic cleavage methods can be used. For analysis of chromatin structure in vivo, base modification, such as methylation of guanines with dimethyl-sulfate, or cleavage reactions, such as DNase I cleavage, have to be carried out within the cellular environment under conditions that retain the integrity of the analyzed structures. After DNA purification, gene-specific fragments are amplified from cleaved genomic DNA by LMPCR as follows (Fig. 1 summarizes the procedure; technical details of the method can be found in ref. 10).

The unique aspect of LMPCR is the ligation of an oligonucleotide sequence onto the 5' end of each DNA molecule. Because a phosphate group is required in this ligation reaction, it is advantageous to use a DNA cleavage method that leaves behind a 5' phosphate group on the sugar phosphate backbone. Cleavage of DNA to generate 5' phosphorylated molecules can be achieved, for example, by the β -elimination step of chemical DNA sequencing.⁽¹¹⁾ Next, primer extension of a gene-specific oligonucleotide (primer 1) generates molecules that have a blunt end on one side. Linkers are li-

gated to the blunt ends to introduce a common sequence to the 5' ends of the sequence ladder fragments. The linker has a 5' overhang to prevent ligation in the wrong orientation. Then, an exponential PCR amplification of the linker-ligated fragments is done using the longer oligonucleotide of the linker (linker–primer) and a second, nested gene-specific primer (primer 2).

After 15–20 cycles of PCR, the DNA fragments are separated on a sequencing gel, electroblotted onto nylon membranes, and hybridized with a gene-specific probe to visualize the sequence ladder.⁽⁵⁾ The single-stranded hybridization probes, which should not overlap the sequence of the second primer by more than 10 bases, can easily be made from an appropriate PCR product by repeated run-off polymerization with *Taq* polymerase using one of the two PCR primers.^(10,12) By rehybridization of the nylon membranes, several gene-specific ladders can be sequentially visualized from one sequencing gel (multiplexing).⁽⁵⁾ Alternatively, amplified fragments can be detected by primer extension using a ³²P-labeled third primer.⁽⁴⁾ Only 1 μ g of mammalian DNA is required per lane of the sequencing gel to obtain good-quality DNA sequence ladders with retention of information on DNA cytosine methylation and in vivo protein–DNA contacts.

LMPCR FOR ANALYSIS OF DNA METHYLATION PATTERNS

It is now well established that methylation of 5'-CG dinucleotides within promoter regions of a gene correlates with a lack of gene expression.^(13–16) LMPCR has been used for sequencing genomic DNA and for determination of DNA cytosine methylation patterns.^(5,7,8,17,18)

FIGURE 1 Outline of LMPCR. A gene-specific oligonucleotide (primer 1) is annealed to genomic DNA fragments that have been obtained by a specific cleavage method, e.g., DNase I treatment or chemical DNA sequencing. This primer is extended with a DNA polymerase to provide blunt ends for ligation of the common linker. Then, a second gene-specific primer (primer 2), which partially overlaps but extends 10–15 bases 3' to primer 1, is used together with the common linker primer (LP) in an exponential PCR reaction. The amplified fragments are separated on a sequencing gel and detected after blotting and hybridization with a single-stranded probe that does not overlap the sequence of primer 2. Alternatively, the amplification products can be visualized by primer extension using a ^{32}P -labeled, third gene-specific primer⁽⁴⁾ and autoradiography of the sequencing gel.

Methylated cytosines are recognized by their failure to react with hydrazine, thus resulting in a gap in the C-specific sequencing ladders. Another useful method for detecting 5-methylcytosine is based on UV-irradiation. 5-Methylcytosine, unlike cytosine, does not participate in formation of pyrimidine(6-4) pyrimidone photoproducts [(6-4) photoproducts].⁽¹⁹⁾ These UV-induced photoproducts occur predominantly at 5'-TC and 5'-CC dinucleotides.⁽²⁰⁾ To detect (6-4) photoproducts at specific nucleotide positions within a gene, UV-irradiated DNA is cleaved at the sites of these photoproducts with hot piperidine, and gene-specific fragments are then amplified by LMPCR.⁽²¹⁾ The absence of a (6-4) photoproduct at, for example, 5'-TCG and 5'-CCG sequences indicates methylation of these sequences.

GENOMIC FOOTPRINTING WITH DIMETHYLSULFATE

Dimethylsulfate (DMS) was the first reagent to be used for *in vivo* footprinting studies⁽²²⁾ and is still the most commonly used one today.^(23–30) DMS is a small molecule that easily diffuses through cell membranes to react with DNA bases in the nucleus. 7-Methylguanines and 3-methyladenines are the predominant modified bases. After treating the cells with DMS, DNA is isolated and cleaved at 7-methylguanines with hot piperidine⁽¹¹⁾ or at 7-methylguanine and 3-methyladenine by heating at neutral pH followed by strand breakage in alkali.⁽²⁸⁾ Gene-specific ladders are then amplified by LMPCR.^(4,7) Differences in the modification patterns between DMS-treated cells and DMS-treated purified DNA, evident as hyperreactivities or protections, indicate *in vivo* protein–DNA contacts or an otherwise altered DNA structure at these sites.

The most significant advantage from using DMS as a footprinting agent is that it can be simply added to the cell culture medium; no manipulation of the cells is required. However, there are some disadvantages: (1) only sequences that contain Gs (or As) will reveal a footprint, (2) DMS can detect protein–DNA contacts only in the major groove of the DNA double-helix, (3) the reagent may penetrate protein–DNA complexes, and (4) since DMS readily reacts with proteins, only those protein–DNA interactions that remain stable after methylation of

the protein factor itself will be indicated. In fact, we found that several footprints at the promoter of the human phosphoglycerate kinase (*PGK1*) gene were difficult to detect with DMS, but were more readily revealed by footprinting with DNase I or UV light.^(9,31)

GENOMIC FOOTPRINTING WITH DNASE I

DNase I is much less base-selective than DMS and, in addition, the footprints from the 31-kD DNase I enzyme are much larger and clearer than DMS footprints. These features have made DNase I the preferred agent for footprinting *in vitro*. Because of its size, DNase I cannot penetrate cell membranes. It can be used on isolated nuclei, but in many cases isolated nuclei do not retain all genuine protein–DNA contacts owing to a leakage of factors during nuclear isolation procedures.^(9,32) Zhang and Gralla recently demonstrated that SV40 minichromosomes in infected cells can be studied by DNase I and micrococcal nuclease treatment of lysolecithin-permeabilized cells.⁽³³⁾ Cells permeabilized by lysolecithin remain intact, display very efficient DNA replication and transcription activities,^(34,35) and should maintain normal nuclear structure much better than isolated nuclei. We adapted LMPCR-aided genomic sequencing for studies of chromatin structure in permeabilized cells using DNase I⁽⁹⁾ and obtained very informative footprints at the promoter of the X-linked *PGK1* gene. Footprints were seen only at the promoter of the active gene, but not when the gene was on the inactive X chromosome. The inactive promoter showed enhanced DNase I cleavage sites that occurred with a periodicity of 10 bp and spanned two nucleosome-size regions. Thus, genomic DNase I footprinting, coupled with LMPCR, proved to be a sensitive method for detecting sequence-specific transcription factors as well as positioned nucleosome-like particles. Figure 2 shows an example of DNase I footprints obtained from permeabilized cells by using LMPCR.

To amplify genomic fragments derived from DNase I cleavage efficiently, a modified LMPCR method was developed. DNase I cleavage results in fragments having 3'-hydroxyl groups. These fragments will contribute to high levels of nonspecific priming events during the

FIGURE 2 Examples of genomic DNase I footprinting and UV photofootprinting. (A) DNase I footprint analysis of the active *PGK1* promoter in permeabilized cells.⁽⁹⁾ Sequences are from the lower strand between nucleotides -30 and -170 . (Lanes 1–3) Maxam–Gilbert-cleaved DNA (G + A, T + C, C) from HeLa cells; (lanes 4 and 5) DNase I-treated naked DNA controls from Y162 cells (containing the active *PGK1* gene); (lanes 6 and 7) DNA from permeabilized Y162 cells treated with DNase I. DNase I footprints are indicated by the open boxes. (B) UV footprint analysis of the *PGK1* promoter along the same sequences.⁽³¹⁾ (Lanes 1–4) Maxam–Gilbert sequencing reactions; (lane 5) DNA from Y162 cells, no UV irradiation; (lanes 6 and 7) distribution of cyclobutane dimers in UV-irradiated purified DNA; (lanes 8 and 9) distribution of dimers in UV-irradiated Y162 cells. Arrows indicate three dipyrimidine sequences that are strongly hyper-reactive in vivo. Note that differences in photoreactivity coincide with DNase I footprints.

LMPCR reactions. This problem was initially circumvented by blocking the 3'-OH groups with a dideoxynucleotide using terminal deoxynucleotidyl transferase.⁽³⁶⁾ More recently, we found that DNase I-cut genomic DNA can be used

directly for LMPCR if fragments of interest are enriched by extension product capture. After extension of a biotinylated gene-specific primer with a DNA polymerase and ligation of the linker, specific fragments are bound to magnetic

streptavidin-coated beads, and most of the genomic DNA is removed by washing the beads. Specifically bound fragments are eluted from the beads in sodium hydroxide and amplified by PCR with a second gene-specific primer and the linker primer. This procedure reduces nonspecific background and improves the clarity of the sequence ladders. The improvements are particularly significant for DNase I-treated DNA samples because most of the small genomic DNA fragments that may contribute to nonspecific priming and amplification events are eliminated prior to the PCR reactions (V.T. Törmänen, G.P. Pfeifer, and A.D. Riggs, in prep.).

IN VIVO FOOTPRINTING WITH UV LIGHT

The use of ultraviolet (UV) light as an agent to detect protein–DNA complexes was pioneered by Becker and Wang.⁽³⁷⁾ Differences in UV reactivity between irradiated purified DNA and irradiated protein–DNA complexes are referred to as “photofootprints.” UV irradiation is probably the least disruptive method to detect DNA-bound proteins in vivo, because intact cells in a Petri dish are simply irradiated from a UV light source for a short period of time. No complex manipulations are necessary.

UV irradiation of DNA produces, in order of their relative frequency, (1) cyclobutane pyrimidine dimers, (2) (6-4) photoproducts, and (3) minor photoproducts, like cytosine hydrates and purine dimers.^(20,38) Cyclobutane dimers are formed between the 5,6 bonds of any two adjacent pyrimidines. (6-4) Photoproducts are characterized by a covalent bond between positions 6 and 4 of two adjacent pyrimidines, mostly between 5'-TpC and 5'-CpC.⁽²⁰⁾ (6-4) Photoproducts in total genomic DNA are formed at a rate of 10–30% of that of cyclobutane dimers,⁽³⁹⁾ but the relative abundance of the two photoproducts strongly depends on the local DNA sequence. LMPCR provides an adequate level of sensitivity for the detection of individual UV-induced DNA photoproducts at the DNA sequence level in mammalian DNA.⁽²¹⁾

Initially, LMPCR has been used for detection of (6-4) photoproducts. (6-4) Photoproducts and their Dewar isomers are sensitive to alkaline cleavage and can be assayed as piperidine labile sites.⁽⁴⁰⁾ Piperidine cleavage of (6-4) photoproducts

ucts results in breaks with a 5'-phosphate group that allows direct amplification by LMPCR.⁽²¹⁾ We also developed a method for mapping of cyclobutane pyrimidine dimers at nucleotide resolution.⁽³¹⁾ Cyclobutane dimers are resistant to alkaline cleavage, but can be detected at the DNA sequence level by cleavage with the cyclobutane dimer-specific endonuclease T4 endonuclease V.⁽⁴¹⁾ This enzyme breaks the glycosylic bond and sugar phosphate backbone at cyclobutane dimers but does not cleave (6-4) photoproducts. Cleavage with T4 endonuclease V leaves behind a dimerized pyrimidine at the 5' end of the damaged strand.^(42,43) These fragments cannot be amplified by the LMPCR procedure directly but require further treatment with *Escherichia coli* photolyase⁽⁴⁴⁾ to split the overhanging pyrimidine dimers at the 5' ends.⁽³¹⁾ The photolyase splitting reaction is complete and sequence-independent, which allows precise mapping of dimer frequencies along any DNA sequence.

Photoproducts in irradiated, purified genomic DNA are distributed with a pattern that is expected from experiments that have used cloned, end-labeled DNA for UV irradiation.^(19,41,45,46) (6-4) Photoproducts are seen preferentially at 5'-TC and 5'-CC sequences and cyclobutane dimers are induced in order of their relative frequency at 5'-TT > 5'-CT = 5'-TC > 5'-CC. However, when the photoproduct spectrum of irradiated purified DNA was compared with that obtained after irradiation of living cells, some striking differences became apparent.⁽³¹⁾ The photoproduct frequency within sequences bound by sequence-specific DNA-binding proteins (transcription factors) was suppressed or enhanced relative to naked DNA or inactive genes, which do not bind these factors. Figure 2 shows an example of these photofootprints displayed at the DNA sequence level by LMPCR mapping. The sequences shown are from the promoter region of the human single-copy gene *PGK1*. It is important to note that the differences in photoreactivity between irradiated purified DNA and irradiated cells fall within the same sequences protected from DNase I in permeabilized cells (Fig. 2). Thus, photofootprints are reliable indicators of sequence-specific protein-DNA contacts in vivo. The most conspicuous differences between irradiated cells and irradiated purified DNA were transcrip-

tion factor-induced photoproduct hot spots within the promoter of the active gene. For example, the dinucleotide sequence 5'-CC within the sequence CCAAT, the binding site for a CCAAT-box binding protein, shows a (6-4) photoproduct signal 30-fold higher when cells with an active *PGK1* gene were irradiated than when purified DNA or cells with an inactive *PGK1* gene were irradiated.⁽³¹⁾ Effects of chromatin structure on UV damage may be most significant in regulatory gene regions as a consequence of binding of transcription factors. In other experiments, the frequency of cyclobutane dimers and (6-4) photoproducts was mapped along exons 1 and 2 of human *ras* proto-oncogenes.⁽¹²⁾ The photoproduct spectra were almost identical between irradiated purified DNA and irradiated cells, indicating absence of UV-detectable DNA binding proteins along the *ras* exon sequences. The theoretical basis for the occurrence of photofootprints has not been clearly defined. After local DNA sequences absorb a UV photon, only those excited bases in a geometry capable of easily forming a photoproduct can photoreact. Adjacent pyrimidines must rotate extensively from their usual B-form DNA alignments to form interpyrimidine covalent bonds. It is conceivable that the bound protein induces an alteration in DNA structure, possibly unwinding or bending, that is favorable or unfavorable for dimer formation.⁽³⁷⁾ Mapping of cyclobutane dimers at the single-copy gene level may also indicate positioned nucleosomes since cyclobutane dimers are modulated with a 10-bp periodicity within nucleosome core DNA.^(47,48)

CONCLUSIONS

LMPCR provides the necessary sensitivity for studies on chromatin structure at nucleotide resolution. When combined with an appropriate in situ footprinting technique, high-resolution mapping of protein-DNA contacts in mammalian cells becomes possible. There are at least three different approaches that can be used for LMPCR-footprinting: treatment of intact cells with DMS, probing permeabilized cells with DNase I, and UV irradiation of intact cells. One or the other method may be more suitable for a particular biological problem. For example, pyrimidine-rich sequences will not give

very informative DMS footprints and purine-rich sequences cannot easily be analyzed with UV light. The most versatile method, although technically slightly more complex than the others, promises to be DNase I footprinting of permeabilized cells. In many cases, the combined use of these methods may provide the most informative and complete picture.

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