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GENOMIC FOOTPRINTING

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INTRODUCTION

The recent development of powerful in vitro methods has led to rapid progress in the characterization of protein factors that bind to conserved sequence motifs within promoter and enhancer elements. By unknown mechanisms they contribute to the observed transcriptional specificity with regard to cell type, developmental timing and environmental responsiveness (1-3). Many of these DNA binding activities can be assayed in crude nuclear extracts (4,5) after binding to their target sequence on a cloned DNA fragment by DNase I footprinting (6) or gel retention (7,8). In the eukaryotic nucleus, however, the genomic DNA is complexed with histones and a large number of non-histone proteins to form a highly compacted and organized structure (9,10). Given the condensed nature of chromatin, it is not obvious that protein-DNA interactions as defined in vitro actually occur in the nucleus of a living cell.

The introduction of the genomic sequencing technology by Church and Gilbert (11) for the first time allowed the direct analysis of protein-DNA interactions in vivo. It employs the use of sequencing gels that permit the separation of genomic DNA fragments with single nucleotide resolution and a sensitive hybridization technique to visualize selectively the region of interest from amongst the rest of the genome. The method allows the detection and accurate mapping of nicks and breaks in the DNA backbone due to enzymatic (e.g., DNase I, S1 nuclease, topoisomerase) or chemical treatment in the context of the complete mammalian genome. Likewise, sites of chemical modification at nucleotides can be monitored if reactions are available that introduce single

or double-stranded breaks at those positions [e.g., the standard sequencing reactions (12)]. While the method was developed initially to reveal methylation at CpG dinucleotides within genomic DNA (11,13,14), its most rewarding application so far has been the direct footprinting of proteins that occupy their target sequences in the nucleus of an intact cell. This "genomic footprinting", following the dimethylsulfate (DMS) reactivity of guanines (15) in intact cells, has been employed successfully to study in vivo protein-DNA interactions in prokaryotes (16,17), yeast (18) and mammals (19-22). One of the most recent studies (22) has indeed revealed that the mere presence of protein factors in nuclei that are capable of binding to cloned DNA fragments in vitro is not sufficient for interaction with their target sequences in vivo. This observation suggests a higher order of regulation, such as changes in chromatin structure or DNA modification, that modulates the access of factors to their binding sites. Genomic footprinting is thus of decisive importance in establishing the biological significance of protein-DNA interactions observed in vitro and addresses the question as to whether ubiquitously present factors have access to DNA in chromatin at their preferred sites of interaction. Furthermore, it permits the characterization of factors that are unstable during extract preparation and purification (e.g., reference 20).

In this chapter we give a detailed description of the methodology involved in genomic footprinting with emphasis upon DMS (dimethylsulfate) reactivity experiments in intact mammalian cells. The experimental conditions described rely on the pioneering work of Church, Ephrussi and Gilbert (11,19,23) with modifications and improvements added more recently (20,22).

GENOMIC FOOTPRINTING

Principle

Crucial to the success of the method is the use of DMS (15), a small chemical that enters the nucleus of an intact cell by diffusion, allowing a direct analysis of proteins binding to DNA in vivo without prior isolation of nuclei. While some results on protein-DNA interactions have been obtained in studies on isolated nuclei both using DMS (23) and DNase I (24,25), it has become obvious from our earlier experiments, as well as published results (e.g., compare 19,23), that protein-DNA interactions are weakened or lost in isolated nuclei, most likely due to leakage of factors during the isolation procedure.

For in vivo analyses intact cells are incubated with DMS under conditions that result in partial methylation of genomic DNA at the N_7-positions of guanines as well as the N_3-position of adenines. For footprinting purposes it is most usual to assay the reactivity of the N_7 residue of guanines (Gs) since it is situated

in the major groove of the DNA that is frequently a site for binding proteins. We assume that if the DNA in the nucleus were homogeneously accessible, all reactive residues would be modified to a similar extent. The tight binding of factors to specific sites, however, will alter the reactivity of Gs. A contact within the major groove will protect the N_7 residue from methylation resulting in a reduced reactivity (Figure 1). Enhanced modification, supposedly the result of locally increased DMS concentration in hydrophobic pockets of the protein (15), is frequently observed as well.

After the methylation reaction the genomic DNA is purified, cleaved with a suitable restriction enzyme and the backbone of the DNA broken at positions of methylated guanines by piperidine (12) (see Figure 1). The highly complex mixture of genomic fragments is then separated on a denaturing polyacrylamide gel with single nucleotide resolution. DNA is electrophoretically transferred

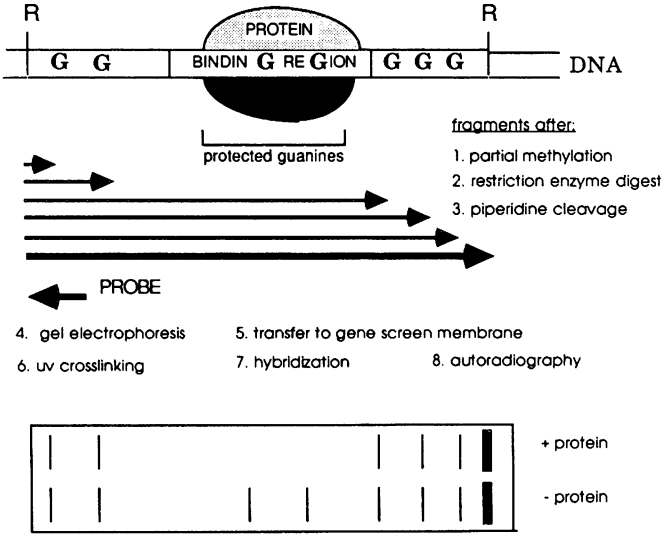

Figure 1. The principle of a genomic footprinting experiment. A protein binding to DNA protects the guanines contained in its binding site from methylation with dimethylsulfate. DNA fragments after the steps 1 to 3 that are indirectly endlabeled and contribute to the genomic guanine sequence are shown. The guanine pattern is visualized on the autoradiograph after steps 4 to 8. Binding of a protein to a specific sequence leads to changes in the reactivity of guanines that are reflected by changes in the intensity of the corresponding bands when compared to control experiments in which the protein does not occupy its binding site. R = restriction site.

onto a nylon membrane and covalently crosslinked to the membrane by UV irradiation. To visualize the genomic sequence of interest, the fragments that contribute to the guanine sequence ladder (Figure 1) are selectively labeled by hybridizing a small single-stranded DNA probe to their ends. This indirect endlabeling of only a small subset of the genomic fragments replaces the direct endlabeling of cloned DNA in *in vitro* footprinting experiments. Autoradiography of the hybridized membrane reveals the guanine-specific sequence ladder of the genomic region to be analyzed. The intensity of each band on the film reflects the reactivity of the corresponding guanine in the cell towards methylation. Comparison of the reactivity of each G with those in control reactions indicates sites of protein contact to the DNA. Figure 2 shows a representative result. About 200 nucleotides of the rat tyrosine aminotransferase (TAT) gene promoter are analyzed. Comparison of the *in vivo* reactivity of each guanine in cells that either do (E) or do not (n) transcribe the TAT gene reveals protected as well as enhanced bands suggesting sites of bound protein in the expressing cells (for details see reference 22).

Problems

The most critical step in genomic footprinting is the hybridization which has to be highly sensitive, yet very stringent. Not only have minute amounts of DNA (in the order of fg/band) to be detected, but this DNA has to be visualized selectively from amongst the large (10^7-fold) excess of total genomic DNA. It is essentially the ratio of specific to unspecific hybridization and membrane background that determines whether an experiment will be successful or not. Here we describe conditions under which a satisfying result is usually achieved.

Further problems involved are related more to biological rather than technical parameters and, thus, differ in every system. The quality of an *in vivo* footprint mainly depends on the complexity of the genome analyzed and on the occupancy of the target site by the DNA binding protein. Heterogeneity in the cell population with regard to the function analyzed due to mixed cell types, cell cycle dependence in unsynchronized cultures or variable responsiveness to certain environmental stimuli will obscure the results. The occupancy of a factor within a cell is also influenced by its abundance and its affinity to the binding sites (on- and off-rates).

The reagent itself, DMS, already presents a certain bias to the study. Clearly, only those binding sites that contain guanines as essential parts will yield a footprint. Furthermore, only those protein-DNA interactions that remain stable after a degree of methylation of the protein factor itself will be monitored successfully. It is quite obvious from these considerations that not every binding protein will be detected equally well, and

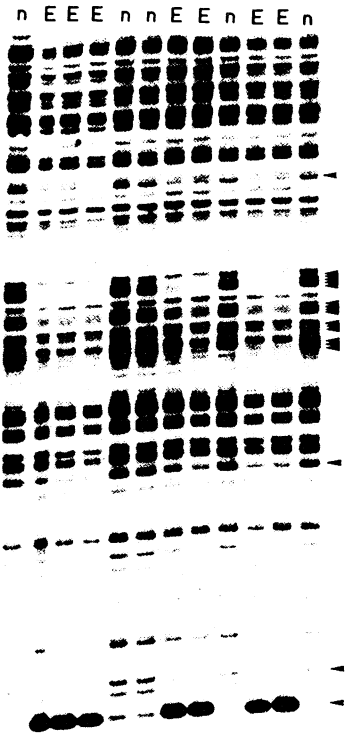

Figure 2. A genomic footprint of the rat tyrosine aminotransferase (TAT) gene promoter in expressing and nonexpressing cells. Each band corresponds to a genomic guanine or a group of unresolved guanines. Comparison of the intensities of each band in cells that either express (E) or do not express (n) the TAT gene reveals guanines of protected and enhanced reactivity (arrows) in the expressing cell (for details, see reference 22).

that a successful genomic footprinting analysis need not reveal every protein-DNA interaction that is occurring in the intact cell.

IN VIVO METHYLATION OF DNA

The parameters of the in vivo methylation reaction such as the number or concentration of cells, the amount of DMS added as well as the exact reaction time appear not to be very critical. It is, however, of importance that all trace of DMS is removed from the sample once the reaction is stopped. For convenience, the methylation is performed on a large number of cells at a time to yield several hundred micrograms of modified DNA. Since

aliquots of this DNA are used for each individual footprint analysis, many distinct genomic regions can be analyzed with DNA of one in vivo methylation reaction.

As DMS is one of the most carcinogenic drugs in laboratory use, care should be taken to inactivate all DMS-containing solutions as well as used plastic ware in 5 to 10 M NaOH.

The following protocol for in vivo methylation results in partial modification of guanine residues equivalent to approximately 1 methylation per 500 base pairs. 1 to 2 x 10^8 mammalian cells are mildly trypsinized and resuspended in 1 ml of complete culture medium (including fetal calf serum) in a 14 ml disposable polypropylene tube (Greiner). They are cooled to about 20°C by short chilling on ice before 5 μl of DMS (Fluka, puriss. p.A.; stored in the dark at 4°C under nitrogen) are added. The reaction is mixed by whirling and incubated for 5 min at 20°C. It is then stopped by addition of 10 ml ice-cold PBS (140 mM NaCl, 2.5 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.5). The tubes are chilled on ice and the cells quickly removed from the DMS-containing solution by centrifugation (5 min, 2.5 krpm, 4°C). The following steps are all performed with ice-cold solutions and cooled (4°C) centrifuges. The cell pellet is washed again with 10 ml PBS. In order to remove DMS trapped in the cytoplasm, nuclei are isolated: the cells are suspended in 1.5 ml of 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 60 mM Tris/Cl pH 8.2, 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM EDTA, 2 mM EGTA, and another 1.5 ml of the same buffer containing 1% Nonidet P40 is added. The sample is mixed well, cell lysis taking 5 min on ice. The nuclei are pelleted (5 min, 3 krpm, 4°C) and washed with 5 ml of the above buffer without sucrose or Nonidet P40. They are resuspended in 1 ml 0.5 M EDTA and sarcosyl (N-lauroylsarcosine, Sigma) and RNAse A are added to 0.5% and 250 μg/ml final concentration, respectively. The RNA is digested for 3 hr at 37°C. Finally, proteinase K is added to 250 μg/ml and the sample is incubated at 37°C overnight.

PREPARING THE DNA FOR A "GENOMIC" BLOT

The genomic DNA after methylation should never be heated above 37°C as this leads to uncontrolled apurination under neutral conditions which results in the appearance of adenine-specific cleavage products in addition to the desired guanine-specific signals. Nevertheless, some degradation due to apurination of the genomic DNA during the purification is unavoidable, but the results of the footprinting gels are usually not adversely affected.

The clear and viscous lysate after the proteinase K treatment is extracted twice with phenol (containing 0.1% (w/v) 8-OH-quinoline and equilibrated with 100 mM Tris/HCl pH 8.0, 10 mM EDTA). Because of the high EDTA concentration in the sample, the organic phase will be above the aqueous one and can easily be removed together with the interphase. The aqueous phase, however,

is the top phase in the subsequent extraction with phenol/chloroform (1:1, equilibrated as above) and is recovered. DNA is dialyzed overnight against 3 liters of TE (10 mM Tris pH 8.0, 1 mM EDTA) at 4°C with one change of buffer. The dialysate is adjusted to 0.3 M sodium acetate and DNA precipitated with ethanol, washed, dried and dissolved in 1 ml TE. The samples are stored frozen at -20°C.

For a genomic blot 30 μg of the DNA samples are cleaved overnight with 2 units/μg restriction enzyme of choice (see below) in a volume of 300 μl. Bovine serum albumin (100 μg/ml, nucleic acid enzyme grade) is included for stabilization of the enzyme. The digest is stopped by adjusting to 10 mM EDTA and 0.3 M sodium acetate, DNA is precipitated and washed with 80% ethanol. The dry pellet is dissolved in 100 μl of 1 M piperidine (Sigma grade 1, freshly diluted in water from a 10 M stock stored in the dark at 4°C). After incubation at 90 to 95°C for 30 min, the reaction is chilled on ice and the solution transferred to a fresh tube. DNA is precipitated with ethanol, washed and dried for at least 2 hr in the speed vac concentrator. The pellet, which may be spread over the tube wall, is resuspended in 20 μl water and dried again for at least one hr (up to overnight). DNA now very easily dissolves in 3 μl of loading buffer [94% deionized formamide (Fluka), 0.05% xylene cyanol, 0.05% bromophenol-blue, 1 mM EDTA pH 8].

CONTROLS

In order to facilitate the unambiguous identification of the genomic sequences, sequence standards should be included. For this purpose cloned DNA corresponding to the region of the genome to be analyzed is cleaved with the restriction enzyme that creates the ends used for indirect labeling and subjected to the standard sequencing reactions (12). Finally, the reaction products are diluted (with formamide loading buffer plus 100 μg/ml sheared salmon sperm DNA) to genome equivalent abundance, taking into account the complexity of the genome (3 x 10^9 bp for mammals) and the intended amount of DNA to be loaded on the gel (usually 30 μg). Thus, for a plasmid of 3 kb size, 30 pg of DNA equals one genome equivalent. These 30 pg are applied to the "genomic" gel in 3 μl loading buffer.

To monitor intrinsic reactivity differences of guanines due to microsequence environment protein-free genomic DNA can also be reacted with DMS *in vitro* following the standard procedure (12). Indeed, not all G-residues are equally reactive towards the chemical, in particular guanines flanked or followed by thymidine residues appear to be hyperreactive. Unfortunately, many of these *in vitro* hyperreactivities are not observed in *in vivo* methylation experiments. This could be explained either by genuine chromatin features or simply by differences in reaction conditions. The reaction conditions *in vitro*, where protein-free DNA in cacodylate

buffer is easily accessible to DMS, and in vivo, where genomic DNA is complexed into chromatin within a nucleus of a living cell and where most of the reagent is likely to be trapped by cytoplasmic proteins, are obviously different. Results of methylation reactivity experiments can therefore be interpreted with greatest confidence if the reactivity patterns obtained from cell types that differ in the particular aspect to be analyzed are compared. Examples of this are cells either treated or not with a certain inducing agent, or different cell types that do or do not express the gene of interest.

The methylated DNA obtained from an in vivo footprinting experiment can be used to analyze several independent loci. Thus, protein-DNA interactions deduced from analyses of a particular site can serve as internal control that helps to interpret negative findings at other sites with confidence if DNA from the same in vivo methylation experiment is used.

PREPARING MEMBRANES FOR HYBRIDIZATION

The separation of the piperidine-treated DNA samples on polyacrylamide gels and the subsequent electroblot of the DNA to the membrane is performed according to routine protocols. The blotting of a sequencing gel of at least 40 cm length, however, needs certain technical attention. Since home-made devices for electroblotting and UV-crosslinking are often used, optimal conditions for the transfer of the DNA to the membrane have to be determined for each setup. Any test designed to reveal optimal transfer conditions should not merely rely on measuring the binding of endlabeled DNA to the membrane, but must also include a hybridization step. Efficient binding of DNA to a nylon membrane does not necessarily guarantee optimal hybridization efficiencies. An extreme case is the Gene Screen plus membrane (NEN) which can retain more than 80% of the transferred DNA after a stringent washing procedure. The DNA, however, is bound so tightly that very little remains available for hybridization. Under the conditions we use, 30 to 40% of the transferred DNA is retained on the Gene Screen (NEN) membrane after hybridization and washing.

We have exclusively used gels of 40 cm length. While other investigators have used gels up to 1 meter length (26), we feel that the additional information obtained with these long gels does not justify the special technical investments demanded.

The DNA samples in loading buffer are denatured for 3 min at 95°C and chilled on ice to avoid reannealing. They are then loaded and separated on a 6% denaturing polyacrylamide gel that is prepared essentially like a standard sequencing gel (with high quality chemicals). It differs from the usual gels in the ratio between acrylamide and bisacrylamide (which is 39:1) and its thickness (1 mm). Before the gel is poured, both glassplates have

to be freshly siliconized as the gel has to be removed for blotting. The preferred electrophoresis buffer is TBE pH 8.8 [10 x TBE = 162 g Tris base (Roth, p.A.), 27.5 g boric acid (Merck) and 9.5 g $Na_2EDTA \times 2\ H_2O$ (Tritiplex III, Merck) per liter]. The gel is prerun at constant voltage (e.g., 900 V for a gel of 30 x 35 x 0.1 cm size) until the current stabilizes. The running conditions should be chosen such that (unlike for sequencing gels) the genomic gel heats up only moderately. It is convenient to load dye markers next to the DNA-containing lanes at intervals to mark the area to be blotted. After sufficient separation of the fragments has been achieved, one glassplate is removed carefully and the gel slowly covered with ashless hardened paper (Schleicher and Schüll; Art. No. 103 00187). The gel sticks tightly to the paper and can easily be removed from the glass plate.

For the transfer of the DNA from the gel to the nylon membrane we use the blotting device supplied by the Harvard Biological Laboratories detailed in reference 11. The Gene Screen membrane, cut to the right size, is homogenously wetted by carefully floating it on the surface of the blotting buffer and submersing. The gel on paper is directly put onto a dry scotch-brite support and then buffer is poured onto the face of the gel. The membrane is laid on the gel. Trapped bubbles have to be squeezed out carefully. The "sandwich" is completed by directly putting the upper scotch-brite support onto the membrane. It is tied tightly with rubber tubing and slowly submersed in a slanted position in the blot buffer. Trapped air bubbles under the scotch-brite can easily be seen and have to be avoided. The blotting buffer is 0.5 x TBE pH 8.3 (10 x TBE = 109 g Tris base, 55 g boric acid and 9.3 g $Na_2EDTA \times 2\ H_2O$ per liter). It has to be changed after each transfer as it is spoiled by urea diffusing from the gel. The blotting conditions that guarantee complete transfer in this device (size 38 x 46 x 20 cm, containing 20 liters of buffer) are 60 min at 90 to 100 volts (3 to 4 A). To avoid undesirable heating of the buffer the transfer is carried out in the cold room (4°C) with precooled buffer. After complete transfer the membrane is air-dried and then baked at 80°C *in vacuo* for 20 min. The side which was in contact with the gel is then irradiated with UV light (254 nm) from the 6 tubes of an inverted transilluminator from which the top panel has been removed. The exact conditions are 20 sec, 20 cm distance, 5000 $\mu Watts/cm^2$. The membrane is now ready for hybridization and can be stored sealed in a plastic bag until use.

PROBES AND PROBE SYNTHESIS

Probing Strategy

The region of the genome that can be analyzed with single nucleotide resolution is limited to about 300 nucleotides by the

resolving capacity of the polyacrylamide gels. The probe used for indirect endlabeling of the genomic fragments contributing to the sequence ladder has to hybridize in close vicinity to the region of interest. Some basic properties of a suitable probe fragment are schematically outlined in Figure 3. Ideally, its size should be between 100 to 150 nucleotides to minimize hybridization to fragments that do not terminate at the proper restriction site and thus do not contribute to the desired sequence ladder (11). It must be free of repetitive sequences, of moderate GC content (probes with GC contents between 35 to 55% have been used successfully) and should not contain strong stop sites for Klenow polymerase (such as long stretches of thymidines) if the strategy suggested here is followed. It should abut the genomic restriction site that creates the end to be labeled by hybridization (compare to Figure 1). In addition to its strategic position in the vicinity of the stretch of DNA to be analyzed, the restriction site (R) itself has to be chosen with care. The corresponding restriction endonuclease has to cleave large amounts of genomic DNA reliably, cheaply and completely (e.g., the enzyme should not be influenced by possible CpG methylation in its recognition sequence). The restriction fragment created has to be long as compared to the probe fragment (larger than 500 nucleotides for good results). Following the procedures described, genomic guanine sequence can be read unambiguously from position 70 from the indirectly labeled end, part of the analyzed sequence overlapping with the probe (Figure 3).

Probe Synthesis

Clearly, a variety of strategies can be followed for efficient incorporation of highly [α^{32}P] labeled nucleotides into a short piece of nucleic acid in order to obtain a probe fragment of high specific activity. Both RNA probes (25) and single-stranded DNA probes (11,20,26) have been used for genomic sequencing. We here describe the method that has initially been suggested by Church and Gilbert (11) and, with modifications (20,22), yields most reliable results in our hands.

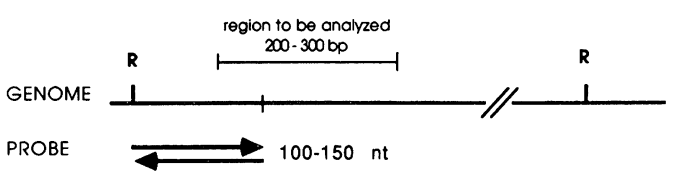

Figure 3. A genomic sequencing probe. Some properties of a suitable probe in context of the genomic region to be analyzed are shown. R: restriction site.

All probe fragments are cloned in either orientation (to label both genomic DNA strands in successive hybridizations) into the M13mp8 vector (27). Large amounts of single-stranded DNA are prepared according to standard procedures. Since a contaminating nuclease activity in some template DNA preparations interfered with probe synthesis, we recommend digestion of the M13 phage particles with proteinase K prior to extensive phenol extractions during purification. The identity and orientation of the insert is verified by direct sequencing of the single-stranded DNA (28).

Figure 4 schematically describes the synthesis of a probe. A universal sequencing primer (e.g., 1211, BRL) is annealed to its complementary sequence in the vicinity of the M13 polylinker. Klenow polymerase is then used to elongate the primer and synthesize the complementary strand of the probe insert which will be highly labeled if [α^{32}P] dATP of high specific activity is included in the reaction. Provided that polymerase, primer and cold nucleotides are in excess, primer elongation will start

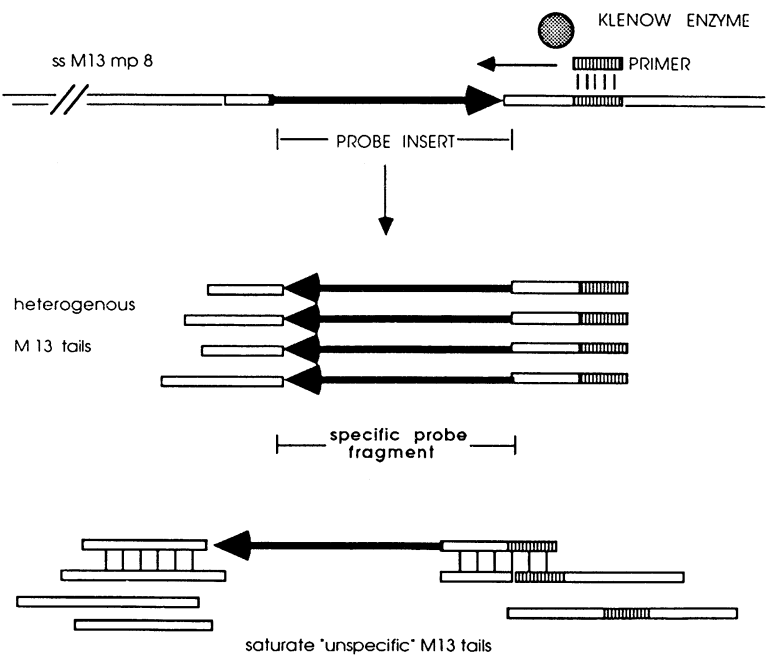

Figure 4. The principle of probe synthesis. The specific probe fragment is cloned into the M13mp8 vector and labeled by elongation of an annealed primer with Klenow polymerase in the presence of [α^{32}P] dATP. Vector-derived portions of the resulting probe fragments are then converted to double strands by hybridization with sonicated complementary M13mp8 wild type DNA in order to reduce the hybridization background.

simultaneously at all available M13 templates. Under these conditions the length of the newly synthesized strands depends primarily on the amount of limiting dNTP (the labeled dATP). Altering the ratio of dATP to template molecules is, therefore, an efficient way of determining the length of the probe fragments (see below and Figure 5). Nevertheless, the reaction products contain varying lengths of sequences complementary to the M13 portion beyond the insert (Figure 4). These M13 "tails" contribute to the number of decays that label a DNA band when a probe fragment hybridizes. They, however, increase both unspecific hybridization and general membrane background. To exclude this latter process the M13 sequences of the isolated single-stranded probe molecules are hybridized with a large excess of cold sonicated complementary M13 wild type DNA (Figure 4). This "shielding" of sequences that do not contribute to specific hybridization greatly improves the results. At the same time the portion of vector-derived sequences contained in probe fragments should be kept minimal to maximize the specific hybridization capacity of the probe. We thus recommend performing a series of analytical probe syntheses with each new batch of template DNA to determine empirically the optimal ratio of single-stranded template DNA to dATP required to obtain probe fragments of suitable lengths.

The result of a series of analytical probe syntheses is shown in Figure 5. The reactions were performed with two template DNAs that contained an identical 110 nt fragment inserted in either orientation. The templates were annealed with primer as described in the following paragraph. Varying amounts of annealed template were then added to a premixed synthesis reaction to yield final ratios of between 15 and 60 molecules dATP per single-stranded template. After the elongation reaction (see below), formamide loading buffer was added, the DNA denatured for 3 min at 95°C and quickly loaded on a 6% denaturing polyacrylamide gel. When the position of the xylene cyanol size marker indicated sufficient resolution of the DNA the gel was directly autoradiographed. The autoradiograph (Figure 5) shows that the average length of the newly synthesized fragments can indeed be tuned by altering the ratio of dATP to template. The fewer dATP molecules per template are offered the shorter the resulting synthesis products will be. The characteristic pattern of fragments visualized by autoradiography corresponds to individual pausing sites for Klenow polymerase, usually at clusters of thymidine residues in the sequence. The pattern of Klenow stops is different in both reactions A and B at those parts where insert sequences are copied. As soon as the synthesis reaches flanking vector sequences (which are identical in both cases), the fragment pattern obtained from the two reactions is identical (above the XC marker). This visible transition helps to define the appropriate conditions to obtain probe fragments that include all specific sequences but minimal vector parts (in this case about 25 pmoles dATP per pmole M13 template).

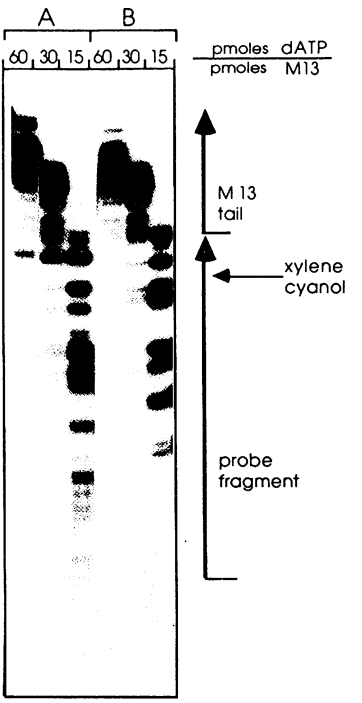

Figure 5. Analytical probe synthesis. A set of analytical probe syntheses with two templates that differ only in the orientation of the inserted fragment was performed. The reaction products were separated on a denaturing polyacrylamide gel and visualized by autoradiography.

Suitable conditions determined by a series of analytical probe syntheses are linearly scaled up for preparative probe synthesis.

A typical example of a probe synthesis is: 1.5 pmoles single-stranded template DNA (~ 3.7 μg) and 3 pmoles of primer (20 ng in 2 μl) are annealed in 100 mM NaCl and 5 mM $MgCl_2$. The volume is adjusted to 20 μl with 10 mM Tris/Cl pH 7.5. The sample is denatured 5 min at 95°C, renatured for 50 min at 50°C and finally put on ice. To the annealed template is added 0.5 μl of 10 mg/ml bovine serum albumin (BRL, nucleic acid enzyme grade), 0.5 μl 200 mM β-mercaptoethanol, 1.5 μl 2 M NaCl, 1 μl of a mix of each 3 mM dGTP, dCTP, dTTP, 1 μl Klenow polymerase (5 units) and 25 to 30 pmoles [α^{32}P]dATP (NEN, 5000 Ci/mmole = 250 μCi). The volume is adjusted to 50 μl with 10 mM Tris/Cl pH 7.5. The reaction is incubated for 30 min at 37°C. Usually more than 90% of the input label will be incorporated. DNA is precipitated with ethanol and the moist pellet after centrifugation and complete

removal of the supernatant is dissolved in 100 μl of formamide loading buffer (as above, but containing 10 mM NaOH).

Most recently, an improved probe synthesis protocol has been developed in our laboratory (F. Weil et al., unpublished data), which employs reverse transcription of a cold RNA template. A detailed description of the method is available upon request.

Probe Purification and Recovery

After the DNA is dissolved in loading buffer and heated for 5 min at 95°C, the sample is quickly loaded into 3 slots (each 1.5 cm wide) of a denaturing polyacrylamide minigel (1 mm thick) that had been heated by pre-electrophoresis. The electrophoresis is stopped when the bromophenol-blue dye has migrated about 4 cm (after about 15 min). One glass plate is removed and the gel covered with Saran wrap. A series of short exposures of the gel (5 to 60 sec) to X-ray film is performed. The slot of the gel should be visible on the longest exposure as a thin line. Ideally most of the labeled DNA should migrate as a broad band above the xylene cyanol dye marker, but separated from the slot by about one centimeter. The parts of the gel that contain most of the labeled material are cut out with a scalpel. No acrylamide closer than 4 mm to the slot should be included to avoid the copurification of M13 template DNA.

The labeled probe DNA is recovered from the acrylamide by a simplified version of the isotachophoresis method (29). The main advantages of this electroelution are high recoveries, the small volume of the recovered material, speed and easy handling which avoids unnecessary exposure to radiation. The eluted DNA is used directly for prehybridization with M13 DNA and subsequent hybridization to the membrane.

The principal setup of the method is schematically outlined in Figure 6. A column (BioRad or equivalent, 10 cm long, 10 mm diameter) is packed to about half column size with sterile Sephadex G50 fine. After equilibration with 10 volumes 40 mM Tris/Cl pH 7.5, the outlet tip (luer filling) is closed with a dialysis membrane held in place by a small ring cut of a luer closing (BioRad column supplementary). Care is taken not to trap any air bubbles. The closed column tip is immersed in a beaker of 40 mM Tris/Cl pH 7.5. After residual buffer is removed from the top of the Sephadex, the gel pieces containing the probe fragments are loaded onto the resin. A droplet of xylene cyanol (or phenol red), which serves as a marker for the migration of the DNA, is added and the column is carefully filled with 100 mM 6-aminocaproic acid. The two electrodes (platinum wire imbedded into plexiglass fittings) are connected: the anode dips into the Tris-containing beaker, the cathode into the caproic acid (Figure 6). 2 to 6 mA (100 to 300 Volts) are applied. The DNA is eluted from the gel and focused with the indicator dye into a sharp band.

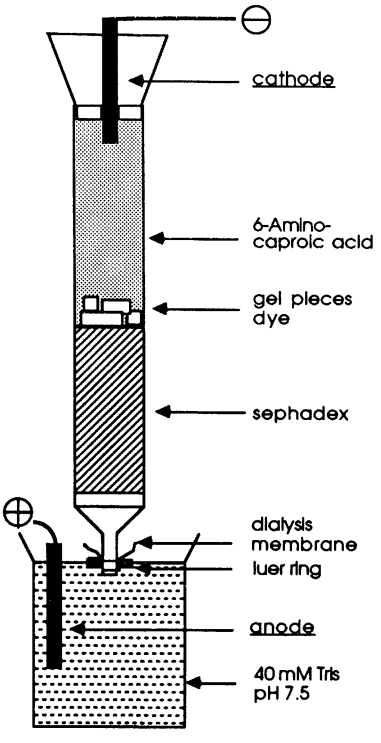

Figure 6. Probe recovery by isotachophoresis.

When elution is complete the band starts to migrate through the column, the peak of the DNA being slightly ahead of the dye marker. Isotachophoresis is stopped when the dye reaches a position 0.5 cm above the base of the column (Figure 6). The caproic acid is completely removed with a pasteur pipette and the dialysis membrane stripped off. The DNA can now be eluted by adding TE to the column and collecting convenient fractions. Usually about 90% of the labeled material is recovered in a volume of 500 to 700 μl. The resulting probe consists of about 50 to 100 ng of specific single-stranded fragments labeled to a specific activity of 4 x 10^9 dpm/μg DNA (we have not used probes for hybridization if less than 2 x 10^8 dpm were recovered). To hybridize vector-derived sequences contained in the probe fragments with cold complementary vector DNA, 20 x SSC (3 M NaCl, 0.3 M Na citrate) is added to a final concentration of 5 x SSC. A 100-fold molar excess of single-stranded wild type M13mp8 DNA that has been extensively sonicated is added giving, typically, a volume of less than 1 ml, and the sample incubated at 65°C for 1 to 2 hr. This prehybridized probe is added directly to the hybridization mix (see below).

HYBRIDIZATION

To visualize femtogram amounts of specific DNA crosslinked to the nylon membrane, the DNA is hybridized to the highly labeled probe synthesized as described above. The sensitivity required necessitates exposure times of a week or longer to visualize the results. Hybridizations and washes thus have to be stringent enough to avoid membrane background even after prolonged exposures, but must still result in maximal labeling of the desired bands. Under the stringent hybridization and washing conditions described, membrane bound genomic fragments smaller than 70 nucleotides will not be labeled.

Very reliable results with respect to both low levels of background and signal intensity are usually obtained when the hybridizations are performed in polypropylene cylinders rotating in an incubator specially adapted for this purpose. The incubator we use, as well as accessory equipment, can be obtained from Bachhofer GmbH (D-7089 Reutlingen, FRG). We usually cut the snout off 100 ml polypropylene measuring cylinders (length 23 cm, diameter 3 cm) and close them with suitable silicone stoppers. Before each hybridization the cylinders have to be cleaned extensively with detergent, 10 M NaOH and water. Care should be taken not to scratch the inner surface of the cylinders by mechanical cleaning.

After UV irradiation the membrane is wetted by floating on 0.5 x TBE, rolled up under buffer and then transferred to the cylinder filled with the same buffer. Extensive overlapping of the rolled-up membrane does not adversely influence the quality of the result obtained. The membrane is manipulated carefully with a thick pipette for smooth attachment to the wall. The buffer is poured off and 20 ml of hybridization buffer are added [7% SDS, BioRad electrophoresis purity reagent; 1% BSA, Sigma A7906; 10 mM EDTA; 0.25 M Na_2HPO_4, pH 7.2. To obtain a solution of 0.5 M Na_2HPO_4, pH 7.2 (1 M Na^+), dissolve 89 g Na_2HPO_4 x 2 H_2O in water and adjust the pH with 85% H_3PO_4 (about 4 ml required). Make up to 1 liter with water]. The cylinder is tightly sealed with a silicone stopper and tape and incubated in the rotating device at 65°C for at least 15 min (up to 2 hr). After this prehybridization the buffer is replaced by 7 ml of hybridization buffer (as above) to which the prehybridized probe has been directly added. Consequently the hybridization parameters are 750 mM monovalent cations, 15 ng/ml or 2.5 to 5 x 10^7 dpm/ml probe DNA, 40 μg/ml sheared single-stranded M13 DNA. The hybridization is allowed to take place for 16 to 24 hr at 65°C.

The buffer used to wash the membrane after hybridization is 20 mM Na_2HPO_4 pH 7.2 (as above), 1 mM EDTA, 1% SDS (Serva). Depending on the GC contents of the probe, 100 mM NaCl are added per 5% of reduced GC contents (from 50%).

Hybridization buffer is poured off and the membrane in the cylinder rinsed 5 times each with 100 ml wash buffer at 65°C. It

is then transferred into a large tray with 1 liter wash buffer at 65°C. For convenience the wash buffer is heated above 70°C, 1 liter poured into a tray on a platform shaker and allowed to cool to 65°C. At this point the membrane is submersed and incubated for 5 min while the buffer slowly cools down. Meanwhile a new liter of buffer is cooled to 65°C in a second tray into which the membrane is transferred. After 8 such washing steps, 5 min each with starting temperature at 65°C followed by cooling, the filters are clean.

The wet filter is sandwiched between two layers of Saran wrap, stretched out on cardboard and exposed to Kodak XAR film with Dupont Cronex Lightning Plus intensifying screen (or equivalent) at -70°C. Care is taken that the DNA-bound side of the membrane is in intimate contact with the film. The signal after a 20 hr exposure is usually sufficient to evaluate the success of the experiment and whether further exposure time is needed. Satisfactory exposures usually take a week or longer depending on the quality of the hybridization.

In most cases it is intended that the hybridized membranes will be reused. To remove the hybridized probe DNA, the membrane is incubated in 1 liter 200 mM NaOH for 15 min at room temperature (on a platform shaker), followed by two washes with 100 mM Na_2HPO_4 pH 7.2, 1 mM EDTA, each 15 min at room temperature. The membrane is now ready for rehybridization.

The suggested treatment with NaOH often helps to get rid of background as well. Sometimes rehybridizations are cleaner than the initial hybridizations. However, with every new stripping of the membrane, approximately 10% of the initial signal is lost.

SUMMARY

In this article we describe a detailed protocol to obtain genomic footprints in mammalian cells. Whether or not the procedure will yield satisfying results will largely depend on the properties of the biological system studied, such as the homogeneity of the cell population with regard to the function analyzed. It may be anticipated that further improvements will be added with the growing number of researchers interested in the methodology. Of importance will be the use of other reagents besides DMS, e.g., UV light (30,31) or camptothecin (32) that penetrate cell membranes to monitor processes and structures in intact cells.

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REFERENCES

1 Dynan, W.S. and Tjian, R. (1985) Nature 316, 774-778.
2 McKnight, S. and Tjian, R. (1986) Cell 46, 795-805.
3 Maniatis, T., Goodbourn, S. and Fischer, J.A. (1987) Science 236, 1237-1245.
4 Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucl. Acids Res. 11, 1475-1489.
5 Wildeman, A.G., Sassone-Corsi, P., Grundström, T., Zenke, M. and Chambon, P. (1984) EMBO J. 3, 3129-3133.
6 Galas, D.J. and Schmitz, A. (1978) Nucl. Acids Res. 5, 3157-3170.
7 Fried, M. and Crothers, D.M. (1981) Nucl. Acids Res. 9, 6505-6525.
8 Garner, M.M. and Revzin, A. (1981) Nucl. Acids Res. 9, 3047-3060.
9 Igo-Kemenes, T., Hörz, W. and Zachau, H.G. (1982) Annu. Rev. Biochem. 51, 89-121.
10 Eissenberg, J.C., Cartwright, J.L., Thomas, G.H. and Elgin, S.C. (1985) Annu. Rev. Genet. 19, 485-536.
11 Church, G.M. and Gilbert, W. (1984) Proc. Nat. Acad. Sci. U.S.A. 81, 1991-1995.
12 Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499-560.
13 Nick, H. and Gilbert, W. (1985) Nature 313, 795-797.
14 Saluz, H.P., Jiricny, J. and Jost, J.P. (1986) Proc. Nat. Acad. Sci. U.S.A. 83, 7167-7171.
15 Ogata, R.T. and Gilbert, W. (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 5851-5854.
16 Nick, H., Bowen, B., Ferl, R.J. and Gilbert, W. (1986) Nature 319, 243-246.
17 Martin, K., Huo, L. and Schleif, R.F. (1986) Proc. Nat. Acad. Sci. U.S.A. 83, 3654-3658.
18 Giniger, E., Varnum, S.M. and Ptashne, M. (1985) Cell 40, 767-774.
19 Ephrussi, A., Church, G.M., Tonegawa, S. and Gilbert, W. (1985) Science 227, 134-140.
20 Becker, P.B., Gloss, B., Schmid, W., Strähle, U. and Schütz, G. (1986) Nature 324, 686-688.
21 Pauli, U., Chrysogelos, S., Stein, G., Stein, J. and Nick, H. (1987) Science 236, 1308-1311.
22 Becker, P.B., Ruppert, S. and Schütz, G. (1987) Cell 51, 435-443.
23 Church, G.M., Ephrussi, A., Gilbert, W. and Tonegawa, S. (1985) Nature 313, 798-801.
24 Jackson, P.D. and Felsenfeld, G. (1985) Proc. Nat. Acad. Sci. U.S.A. 82, 2296-2300.
25 Zinn, K. and Maniatis, T. (1986) Cell 45, 611-618.
26 Saluz, H. and Jost, J.P. (1986) Gene 42, 151-157.
27 Messing, J. and Vieira, J. (1985) Gene 19, 269-276.

28 Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Nat. Acad. Sci. U.S.A. 74, 5463-5467.
29 Öfverstedt, L.G., Hammarström, K., Balgobin, N., Hjerten, S., Petterson, U. and Chattopadhyaya, J. (1984) Biochim. Biophys. Acta 782, 120-126.
30 Sellec, S.B. and Majors, J. (1987) Nature 325, 173-177.
31 Becker, M.M. and Wang, J.C. (1984) Nature 309, 682-687.
32 Stewart, A.F. and Schütz, G. (1987) Cell 50, 1109-1117.