

Methods in Enzymology

Volume 218

RECOMBINANT DNA

Part I

METHODS IN ENZYMOLOGY

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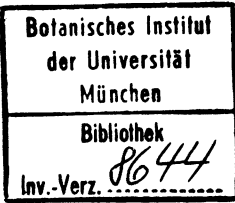
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Preface

Recombinant DNA methods are powerful, revolutionary techniques for at least two reasons. First, they allow the isolation of single genes in large amounts from a pool of thousands or millions of genes. Second, the isolated genes from any source or their regulatory regions can be modified at will and reintroduced into a wide variety of cells by transformation. The cells expressing the introduced gene can be measured at the RNA level or protein level. These advantages allow us to solve complex biological problems, including medical and genetic problems, and to gain deeper understandings at the molecular level. In addition, new recombinant DNA methods are essential tools in the production of novel or better products in the areas of health, agriculture, and industry.

The new Volumes 216, 217, and 218 supplement Volumes 153, 154, and 155 of *Methods in Enzymology*. During the past few years, many new or improved recombinant DNA methods have appeared, and a number of them are included in these new volumes. Volume 216 covers methods related to isolation and detection of DNA and RNA, enzymes for manipulating DNA, reporter genes, and new vectors for cloning genes. Volume 217 includes vectors for expressing cloned genes, mutagenesis, identifying and mapping genes, and methods for transforming animal and plant cells. Volume 218 includes methods for sequencing DNA, PCR for amplifying and manipulating DNA, methods for detecting DNA-protein interactions, and other useful methods.

Areas or specific topics covered extensively in the following recent volumes of *Methods in Enzymology* are not included in these three volumes: "Guide to Protein Purification," Volume 182, edited by M. P. Deutscher; "Gene Expression Technology," Volume 185, edited by D. V. Goeddel; and "Guide to Yeast Genetics and Molecular Biology," Volume 194, edited by C. Guthrie and G. R. Fink.

RAY WU

METHODS IN ENZYMOLOGY

VOLUME I. Preparation and Assay of Enzymes

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VOLUME II. Preparation and Assay of Enzymes

Edited by SIDNEY P. COLOWICK AND NATHAN O. KAPLAN

VOLUME III. Preparation and Assay of Substrates

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[40] Footprinting of DNA-Binding Proteins in Intact Cells

By PETER B. BECKER, FALK WEIH, and GÜNTHER SCHÜTZ

Introduction

The molecular analysis of protein factors that regulate important processes in the cell nucleus, such as transcription, replication, and recombination, has intensified over recent years. The mechanism of action of one class of protein factors (exemplified by transcription factors) requires their specific binding to short DNA sequence modules at regulatory loci in the genome.¹⁻³ Powerful techniques such as DNase I footprinting⁴ have been developed to demonstrate specific binding of purified proteins or components of crude nuclear extracts to DNA *in vitro*. The DNA in these assays usually is of low complexity and easily accessible to the protein to be studied. Genomic DNA in the nucleus of eukaryotes, however, is of high sequence complexity and associated with histones and nonhistone proteins to form the hierarchical structures of chromatin.⁵ The density of structural protein components associated with DNA in the nucleus and its resulting highly compacted state raise questions as to whether the proteins that bind to naked DNA *in vitro* will have access to their potential binding sites in chromatin. It is of particular interest in this context, whether chromatin structure contributes to the mechanisms of regulation by modulating the accessibility of factors to their binding sites in response to extracellular stimuli.⁶

The development of genomic footprinting enabled the direct visualization of specific protein-DNA interactions within the living cell.⁷ The refinement of the indirect end-labeling technique^{8,9} by Church and Gilbert,¹⁰ along with an improved hybridization protocol and single-nucleotide resolution of stretches of genomic DNA, allows the mapping of highly complex genomic DNA. Although originally designed to obtain genomic sequence

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⁷ A. Ephrussi, G. M. Church, S. Tonegawa, and W. Gilbert, *Science* **227**, 134 (1985).

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⁹ S. A. Nedospasov and G. P. Georgiev, *Biochem. Biophys. Res. Commun.* **92**, 532 (1980).

¹⁰ G. M. Church and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991 (1984).

information and to map the occurrence of modified nucleotides,¹⁰ the protocols were immediately applicable for footprinting experiments in the context of the entire genome; the direct labeling of a cloned DNA fragment used in an *in vitro* experiment is replaced by indirect end-labeled probing, which visualizes the sequence of interest selectively.^{7,11}

The methods we will describe in detail are refinements of the original protocol^{7,10} and have been found to reproducibly yield *in vivo* footprints of high quality from mammalian genomes. The importance of the genomic footprinting approach is highlighted in a study¹² of a series of ubiquitous proteins that interact with sequences upstream of the rat tyrosine amino-transferase (TAT) gene *in vitro*. While all factors were present in the nuclei of cells whether they did or did not express the TAT gene, productive interaction of the proteins with their potential binding sites was observed only in the TAT-expressing hepatoma cells.¹² Because all binding sites in the chromatin of the TAT-expressing cells, but not of the nonexpressing cells, are within regions that are hypersensitive to DNase I, these results may document an involvement of chromatin components in the determination of binding site accessibility. Occlusion of proteins from potential binding sites *in vivo* may also be due to CpG methylation in mammalian DNA. Binding of a number of transcription factors has been shown to be sensitive to modification of CpG residues in their recognition sites.^{13,14} Genomic sequencing can be used to obtain important information about the methylation status of CpGs in the nucleus and—in combination with *in vivo* footprinting—their effects on protein interaction.^{12,15} Genomic footprinting has yielded valuable information about the mechanisms of action of well-known transcriptional activator proteins. For example, although the interaction of steroid receptors with their response elements *in vivo* is dependent on the presence of the hormone,^{16–18} purified glucocorticoid and progesterone receptors can interact with their binding sites *in vitro* in the absence of hormones.^{19,20} Binding of a factor to the metal-responsive element of the metallothionein promoter was also observed

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only after metal induction.^{21,22} In contrast, a cAMP-responsive element (CRE) located within the tissue-specific enhancer of the TAT gene is contacted by protein even in uninduced hepatoma cells, reflecting the basal state of protein interaction at this CRE and the basal level of transcription. A detailed genomic footprinting analysis monitoring protein interaction at the CRE during cAMP stimulation revealed a transient modulation of binding activity correlating with transcriptional induction. This led to the conclusion that activation was brought about by a cAMP-dependent posttranslational modification of the factor that interacts with the cAMP response element.^{23,24} Protein binding at the serum response element in the control region of the *c-fos* gene is observed before and after serum induction.²⁵ In this case protein-protein interactions or posttranslational modifications of the DNA-binding protein may be required for transcriptional activation.

In summary, genomic footprinting is the method of choice to study (1) the biological relevance of protein-DNA interactions previously established *in vitro*, (2) the binding sites of factors in order to identify potential sites for functional analysis, (3) the accessibility of target sites in chromatin, (4) the mechanisms of action of DNA-binding proteins, (5) the action of factors that are unstable during purification, and (6) the occurrence of CpG methylation at specific sites.

Principle

The genomic footprinting methodology can be subdivided into three distinct steps: (1) a chemical reaction performed with intact cells to randomly modify nuclear DNA under conditions in which DNA-binding proteins protect their binding sites from the modification; (2) purification and single nucleotide resolution of the genomic DNA; and (3) visualization of the actual footprint. We will focus in this section on the strategy that has been successfully applied in the studies mentioned above: the use of dimethyl sulfate (DMS) as a reagent to chemically modify DNA *in vivo* and the indirect end labeling by hybridization to visualize the footprint. An alternative detection method that employs polymerase chain reaction

²¹ R. D. Andersen, S. J. Taplitz, S. Wong, G. Bristol, B. Larkin, and H. R. Herschman, *Mol. Cell. Biol.* **7**, 35 (1987).

²² P. R. Müller, S. J. Salser, and B. Wold, *Genes Dev.* **2**, 412 (1988).

²³ F. Weih, A. F. Stewart, M. Boshart, D. Nitsch, and G. Schütz, *Genes Dev.* **4**, 14 (1990).

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²⁵ R. E. Herrera, P. E. Shaw, and A. Nordheim, *Nature (London)* **340**, 68 (1989).

(PCR) amplification of the genomic sequences has been described^{26–29} and will be discussed in the final section of this chapter. Alternative routes can also be followed to create the footprint on the genomic DNA. Dimethyl sulfate has been widely used as a modifying agent for DNA because of its small size and hydrophobic character, which enable it to diffuse through the cellular and nuclear membranes of intact cells. Various nucleases that are often used in *in vitro* footprinting experiments cannot enter intact cells and thus will work only on permeabilized cells or isolated nuclei. If leaking of proteins from nuclei is carefully minimized genomic footprints can also be obtained with nucleases.^{30–33}

Figure 1 summarizes the technical steps (1)–(9) involved in the procedure detailed below. In step (1) of Fig. 1 a hypothetical protein–DNA interaction in a nucleus is schematized. The binding site for the protein contains a guanine (G) residue that is contacted by the protein. Protein binding also distorts the nearby DNA structure, symbolized by the adjacent G residue. Cells in suspension are treated with DMS, which diffuses into the nuclei and methylates the N-7 position of guanines as well as the N-3 position of adenines [Fig. 1, step (2)]. The conditions are chosen such that a partial methylation of guanine residues is achieved as in the G-specific sequencing reaction in the Maxam–Gilbert protocol.³⁴ The N-7 position of guanine is directed toward the major groove of the DNA and is frequently contacted by sequence-specific DNA-binding proteins. A guanine in the recognition sequence of a protein will be protected from methylation in the presence of bound protein and thus methylated at reduced frequency as compared with neighboring guanines. Protein binding to DNA can also result in hyperreactivity of guanines toward DMS. This may be caused either by a distortion of the DNA structure in the vicinity of a bound protein [see also Fig. 1, step (1)], or by a locally increased concentration of DMS due to its trapping in hydrophobic pockets of the protein.³⁵ To distinguish DMS reactivity differences of guanines caused by protein binding from those due to the microsequence environ-

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³⁵ R. T. Ogata and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5851 (1978).

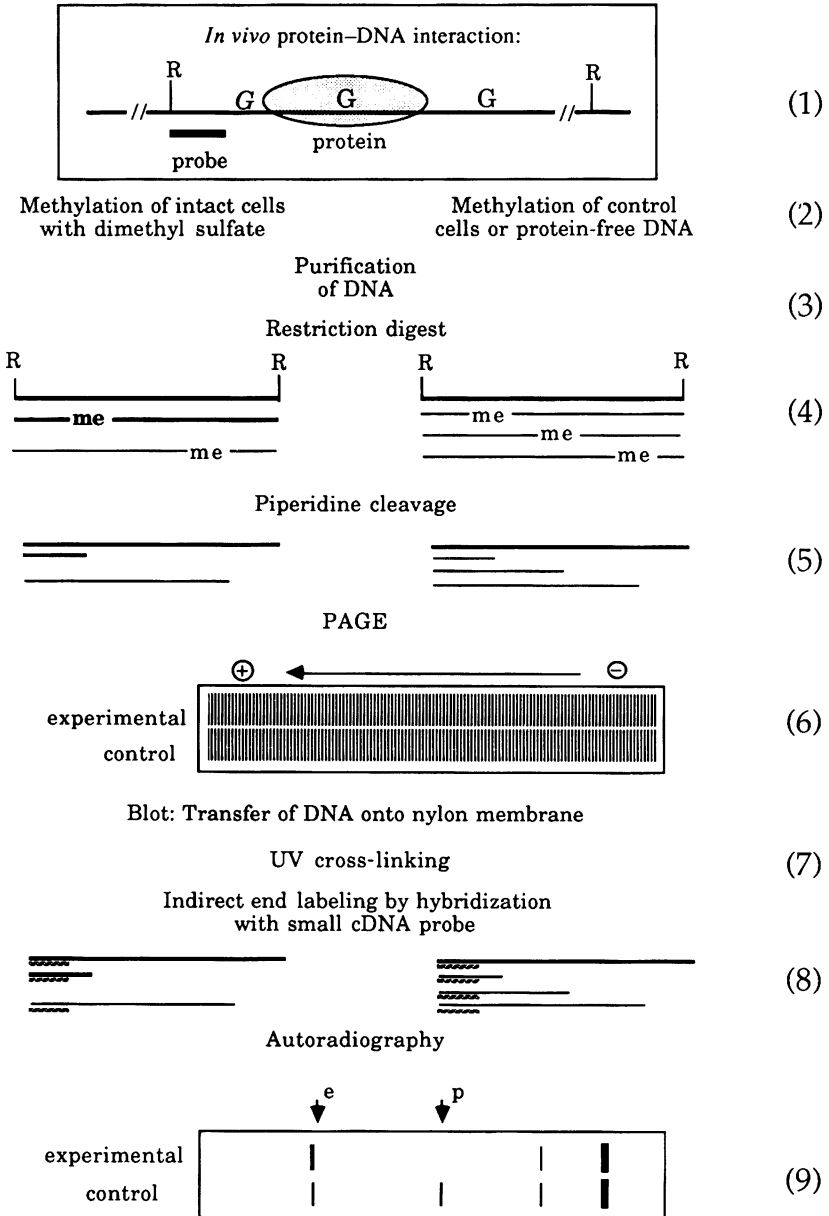


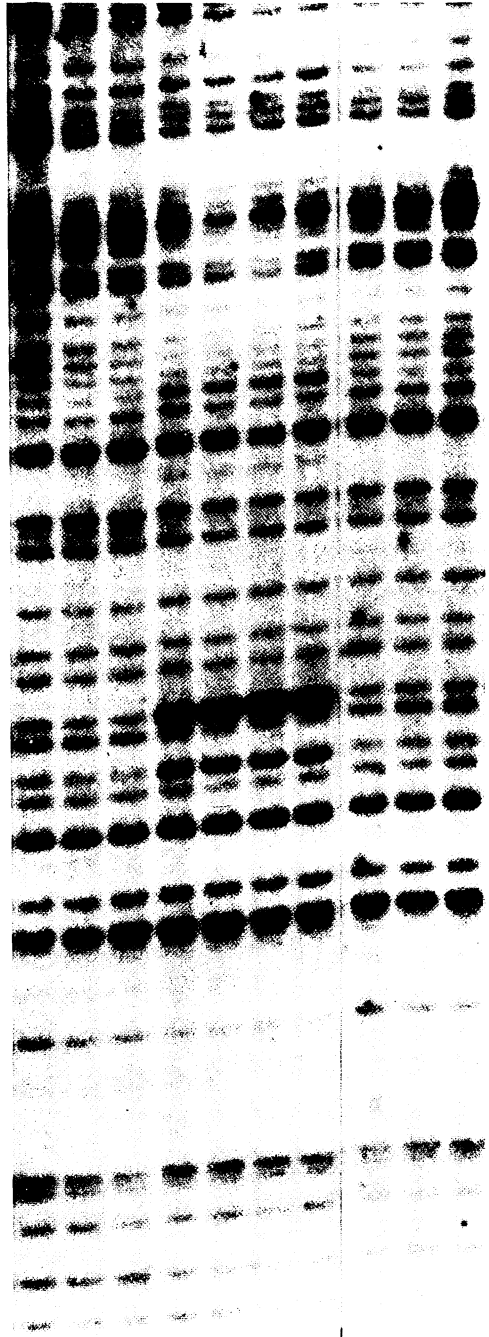
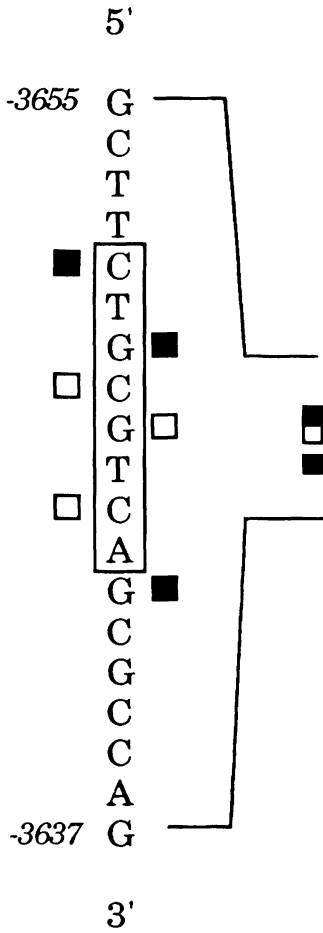
FIG. 1. A schematic outline of the procedures involved in the *in vivo* footprinting methodology. Steps (1) to (9) are referred to in text.

ment, control reactions are carried out in parallel on cells that serve as a control for the effect to be observed (e.g., cells that were not treated with an inducing agent or cells that do not express the gene under study). If such control cells are not available, protein-free genomic DNA can be methylated as a standard. This is, however, not the ideal control because it is conceivable that guanine residues in chromatin react differently with DMS from those in protein-free DNA *in vitro*.¹⁶

After the methylation reaction has been terminated, genomic DNA is isolated and then cleaved with a restriction enzyme to create the fragments needed for indirect end labeling analysis [Fig. 1, step (3)]. The DNA sequence to be analyzed is represented in the genomic DNA pool as a family of fragments [Fig. 1, step (4)]. Due to the partial methylation reaction, many fragments will carry only one methylated guanine. Most of the guanine residues in the nucleus will be similar in DMS reactivity and fragments with the corresponding methylated G's will be found at approximately equal frequency. If guanines were protected from modification, the fragment with a methylated G at the corresponding position will be missing from the pool. Similarly, a fragment with a DMS-hyperreactive guanine will be overrepresented in the pool as compared to the DNA from the control reaction [Fig. 1, step (4), compare left- and right-hand sides].

To map the positions and frequency of methylated guanines by indirect end labeling, the modification must be converted into a break in the DNA backbone [Fig. 1, step (5)]. This is achieved using piperidine, following standard sequencing procedures.³⁴ The complex mixture of DNA fragments resulting from restriction enzyme digest and piperidine cleavage at sites of methylated guanines is now separated with single-nucleotide resolution on an acrylamide gel [Fig. 1, step (6)]. The DNA is then transferred from the gel onto a nylon membrane to which it is covalently cross-linked with UV [Fig. 1, step (7)]. From among the vast excess of unrelated DNA sequences on the membrane, the fragments of interest are now visualized by hybridization of a small DNA probe that abuts the end set by the restriction enzyme [see also Fig. 1, step (1)]. The indirect end label thus highlights the family of fragments that share the end to which the probe hybridizes but differ in the other end, which is determined by the position of the methylated guanine [Fig. 1, step (8)]. The labeled bands are detected by autoradiography. Each band on the film corresponds to the position of a guanine (or a group of unresolved guanines) in the genomic DNA. The intensity of each band is correlated with the reactivity of the corresponding G residue toward methylation *in vivo*. Comparison of the intensity of each band in the experimental DNA with the one from the control reaction allows identification of the guanines within or close to DNA-binding sites of proteins in the cell. Both the reduced intensity of a

XC | FTO-2B | HTC



band [Fig. 1, step (9), protection] or an increased intensity [Fig. 1, step (9), enhancement] are indicative of protein–DNA interaction.

Figure 2 shows an example of a genomic footprinting experiment. A pattern of strong enhancements and weaker protection is caused by protein binding to a functional CRE within the cAMP-responsive enhancer of the TAT gene in FTO-2B hepatoma cells. CREB protein binding to this sequence is neither detected in fibroblast cells, in which the gene is not expressed, nor in hepatoma (HTC) cells, in which the gene can no longer be induced by cAMP (see Refs. 22 and 23 for further details).

Procedures

Each section begins with a list of reagents and materials needed, followed by a basic description of the procedure and finally a section with additional comments, hints, and background information. High-quality chemicals are used throughout the procedures.

In Vivo Methylation of Cells

Materials

Dimethyl sulfate (DMS): Fluka (Ronkonkoma, NY), puriss. p.A., stored in the dark at 4° under nitrogen; DMS is a potent carcinogenic agent and thus all solutions containing DMS as well as used plasticware should be inactivated with 5–10 M NaOH

Phosphate-buffered saline (PBS): 140 mM NaCl, 2.5 mM KCl, 8.1 mM NaH₂PO₄, 1.5 mM KH₂PO₄, pH 7.5

Nuclei buffer: 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 60 mM Tris-HCl (pH 8.2), 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM ethylene glycol-bis (β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA)

FIG. 2. A typical result of an *in vivo* footprinting experiment: Protein interaction at a cAMP response element within the tissue-specific enhancer of the tyrosine aminotransferase (TAT) gene (Ref. 22). The binding of a protein is inferred from prominent enhancements (closed boxes) and a weak protection (open box) that correlate with cAMP induction of TAT transcription. Control cells, such as a fibroblast cell line (XC) in which the gene is not expressed, or HTC hepatoma cells, in which the cAMP induction is impaired, do not show protein binding in chromatin. The changes on the upper strand, visible on the autoradiogram, are marked on the right-hand side of the sequence, enhancements and protections on the lower strand (not shown; see Ref. 22) are indicated to the left of the sequence. The TAT CRE sequence is boxed.

Nonidet P-40

Sarkosyl: *N*-Lauroylsarcosine (Sigma, St. Louis, MO), 20% (w/v)

Procedure

Cells are mildly trypsinized and resuspended in 1 ml of the original medium at 25° in a 14-ml polypropylene tube. To this 5 μ l of DMS is added, and the reaction is mixed by swirling and incubated for 5 min at 25°. The reaction is stopped by the addition of ice-cold PBS to fill the tube and chilled on ice. The following steps are performed at 4° without delay. Cells are collected by centrifugation in a cooled centrifuge (5 min, 2500 rpm), resuspended in 10 ml of cold PBS, and pelleted as before. The cells are resuspended in 1.5 ml nuclei buffer and an equal volume of nuclei buffer containing 1% (v/v) Nonidet P-40 is added, thoroughly mixed, and incubated on ice for 5 min, during which cell lysis occurs. Nuclei are recovered by centrifugation (5 min, 3000 rpm), washed with 5 ml nuclei buffer without sucrose or Nonidet P-40, and lysed by suspension in 1 ml 0.5 M EDTA with vigorous mixing. Sarkosyl and RNase A are added to 0.5% (w/v) and 250 μ g/ml, respectively. After 3 hr of incubation at 37° proteinase K is added to a final concentration of 250 μ g/ml and the incubation is continued at 37° overnight.

The above protocol usually results in a partial methylation of 1 in 500 base pairs in the genomic DNA. The reaction is dependent on the DMS concentration in the nuclei, which in turn is determined by the poor solubility of the hydrophobic chemical in water. Increasing the amount of DMS or the incubation times does not influence the reaction significantly and thus suitable guanine ladders are obtained under a variety of conditions. Once the reaction is stopped the chemical must be removed as completely as possible by thorough washes of the cells and nuclei. Traces of DMS in the following overnight incubation at 37° will continue to react with the now protein-free DNA and obscure potential footprints.

Although the volume of the cell suspension should not be varied, the actual cell number is not critical to obtain the desired partial modification. Between 4×10^7 and 2×10^8 cells have been methylated with satisfactory results. Depending on the availability of the cells, it is convenient to process a larger number of cells at a time to obtain several hundred micrograms of methylated DNA. This DNA can be used for many experiments to visualize proteins interacting with chromatin at different sites. Thus the presence of a footprint at one specific site can serve as an "internal control" for the results of a new site obtained from the same methylated genomic DNA. If, for example, a clear effect of an inducing agent is seen at one particular genomic location when methylated DNAs

from induced cells and control cells are compared, the absence of such effects at another site can be interpreted with confidence.

Preparing DNA Samples for Electrophoresis

Materials

Phenol: Contains 0.1% (w/v) 8-hydroxyquinoline; equilibrate with 100 mM Tris-Cl (pH 8.0), 10 mM EDTA

Phenol-chloroform (1 : 1): Equilibrate as above

TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA

Bovine serum albumin (BSA): Nucleic acid enzyme grade, 10 mg/ml

Piperidine [10 M stock, grade 1 (Sigma)]: Store at 4° in the dark; before use dilute freshly to 1 M with water

Loading buffer: 94% (w/v) deionized formamide (Fluka), 0.05% (w/v) xylene cyanole, 0.05% (w/v) bromphenol blue, 10 mM EDTA

Procedure

The clear and viscous DNA solution after proteinase K treatment (see above) is diluted by addition of 1 ml 0.5 M EDTA and extracted twice with phenol. The high EDTA concentration renders the aqueous phase more dense than the organic phase, allowing easy removal of organic phase together with the interphase. In the subsequent extraction with phenol-chloroform, the aqueous phase is the upper phase again. The DNA is dialyzed overnight against 3 liters of TE buffer at 4° (one change of buffer). To the dialysate add 1/10 vol of 3 M sodium acetate (pH 7), followed by 2.5 vol of ethanol. The precipitate is collected by centrifugation, washed with 80% ethanol, dried in a desiccator, and finally redissolved in 1 ml of TE. The DNA is stored frozen at -20°. The concentration of each sample should be determined by OD measurement. The DNA is analyzed by electrophoresis on a 0.6% (w/v) agarose gel.

Genomic DNA purified by this procedure is sufficiently clean to be easily digested with the restriction endonuclease [see Fig. 1, step (4) and below] to create the fixed end for the indirect end labeling. Thirty micrograms of each DNA sample is digested with 60 units of restriction enzyme overnight at 37° in a volume of 300 μ l. Bovine serum albumin is added to 100 μ g/ml for stabilization of the enzyme. The reaction is stopped by adding EDTA to 10 mM and sodium acetate (pH 7) to 0.3 M and the DNA is precipitated with 2.5 vol ethanol. After chilling, the DNA is collected by centrifugation (10 min, Eppendorf table centrifuge), washed with 80% ethanol, and dried in a vacuum concentrator (SpeedVac; Savant, Hicksville, NY). Pellets are dissolved in 100 μ l 1 M piperidine and incu-

bated at 85–90° for 30 min [Fig. 1, step (5)]. After chilling on ice, the solution is transferred into a fresh tube and precipitated as above. The DNA, which usually is spread over the tube wall as a film, is carefully dissolved in 100 μ l of water and dried for 2 hr in a vacuum concentrator. The pellets are dissolved in 20 μ l of water and dried again for at least 1 hr (or overnight). The resulting fluffy pellet is dissolved in 3 μ l loading buffer.

Methylated DNA should not be heated above 37° prior to the piperidine reaction in order to avoid uncontrolled depurination in the neutral buffer conditions. DNA that appears to be larger than 10 kb when analyzed on a 0.8% agarose gel will be suitable for a genomic footprinting experiment.

Preparing Membranes for Hybridization

Materials

GeneScreen membrane (Du Pont/NEN, Boston, MA)

Electrophoresis buffer (TBE, pH 8.8) (10 \times stock solution): 162 g Tris base, 27.5 g boric acid, 9.5 g Na₂EDTA \cdot 2H₂O/liter

Ashless hardened paper (Cat. No. 103 00187; Schleicher & Schull)

Blotting buffer (0.5 \times TBE, pH 8.4) (10 \times stock solution): 109 g Tris base, 55 g boric acid, 9.3 g Na₂EDTA \cdot 2H₂O/liter. Due to the diffusion of urea from the gel into the blotting buffer, the buffer must be changed before every use

Procedure

The piperidine-treated genomic DNA is now separated on a denaturing polyacrylamide sequencing gel [Fig. 1, step (6)]. We routinely run wide gels (30 \times 35 cm), which differ from common sequencing gels in their thickness (1 mm) and the cross-linking ratio (acrylamide : bisacrylamide, 39 : 1). Prior to pouring of the gel both glass plates must be freshly siliconized to facilitate the later blotting of the gel. Gel and electrophoresis buffer is TBE, pH 8.8. The gel is prerun at a constant 900 V for about 3 hr, until the current stabilizes [or, for convenience, overnight (~12 hr) at 220–250 V], followed by 30 min at 900 V. The DNA samples in loading buffer are denatured for 3 min at 85–90°, chilled on ice, and loaded on the gel. Electrophoresis is carried out at a constant 900 V (under those conditions the gel does not heat up) until the desired resolution is achieved. From time to time during the run, dye markers are loaded into the slots adjacent to the samples to mark the area to be blotted. After the run, the glass plates are carefully separated and the gel covered with a sheet of ashless hardened paper to which it sticks tightly and thus can be removed from the supporting plate [do not use Whatman (Clifton, NJ) 3MM paper].

The apparatus used for electroblotting is a replica of the Harvard Biological Laboratories model detailed in Ref. 10. Its size ($38 \times 46 \times 20$ cm, holding 20 liters of buffer) accommodates a wide sequencing gel. Two spirals of platinum wire at both the bottom and the lid constitute the two electrodes. A supportive plastic grid with feet is covered with a spongy pad. The gel supported on paper is placed directly on the dry pad and its surface wetted with blotting buffer. The GeneScreen membrane, prewetted in the same buffer, is placed on top of the gel and trapped bubbles are squeezed out carefully. The membrane should be marked so that identification of the side that is in contact with the gel is possible. A second layer of dry spongy pad is directly put on top of the membrane, and another plastic grid with feet completes the "sandwich," which is tied together with rubber tubings. The sandwich is flipped over (thus blotting will be downward) and *slowly* submerged into the blotting buffer in a slanted position. It is important to make sure that no air bubbles are trapped underneath the spongy pad, which would cause severe blotting artifacts. The electrodes are connected such that the anode is at the bottom of the tank (and itself covered by a spongy pad). Thus gas bubbles are not trapped in the sandwich. The transfer is performed in the cold room with precooled buffer. It is complete after 1 hr of blotting at constant voltage of 90–100 V (3–4 A). The membrane is now air dried on a filter paper and baked in a vacuum oven at 80° for 20 min. Next, the side of the membrane that was in contact with the gel is irradiated with UV light (254 nm) [Fig. 1, step (7)] to cross-link the DNA covalently to the nylon matrix. A variety of devices can be used for irradiation, some of which are commercially available. We use an inverted transilluminator from which the quartz glass panel is removed and irradiation comes from six bulbs at a distance of 20 cm for 20 sec ($5000 \mu\text{W}/\text{cm}^2$). The membrane is now ready for hybridization.

We advise that the conditions for UV cross-linking be carefully optimized for each individual device used. With GeneScreen membranes optimal hybridization signals are obtained under cross-linking conditions that result in the retention of 30–40% of the blotted DNA on the membrane after hybridization and wash. Tighter cross-linking adversely affects its base-pairing capacity to the DNA probe during hybridization.

The spongy pads of the blotting device should not be stored in the tank under blotting buffer, but rather separately, as it needs to be dry when the gel is placed on it for blotting. When the pads are wet, air bubbles cannot escape through the meshes when the sandwich is submerged in the buffer, thus causing blotting artifacts.

To identify the genomic sequence unambiguously, sequence standards produced with cloned DNA should be run alongside the genomic samples.

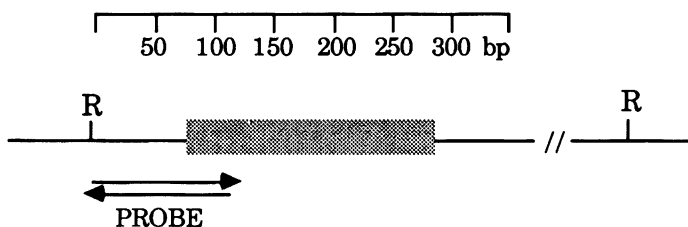


FIG. 3. Probing strategy. The probe abuts the strategic restriction site (R) and partially overlaps the genomic region that can be scanned for protein-DNA interaction with good resolution (shaded field). See text for details.

The plasmid standards must be diluted to a concentration that approaches the amount of specific sequence in a genomic sample to yield a signal of roughly equal intensity following hybridization. A plasmid containing the cloned sequence to be analyzed in the genome is cleaved with the restriction enzyme that is also used to cut the genomic DNA and then subjected to Maxam-Gilbert sequencing reactions.³⁴ The reaction products after piperidine cleavage are diluted into loading buffer (including 100 $\mu\text{g}/\text{ml}$ sheared salmon sperm DNA) to genome equivalent abundance, taking into account the complexity of the genome (3×10^9 bp for mammals) and the absolute amount of processed genomic DNA to be loaded on the gel (30 μg). Thus for a plasmid of 3 kb size, 30 μg of DNA equals one genome equivalent. Thirty picograms is applied to the sequencing gel in 3 μl of loading buffer. Plasmid dilutions can be used to evaluate the sensitivity of the analysis and provide useful standards to monitor improvements during subsequent experiments.

Probing Strategy

To visualize interactions of protein at one particular site in the genome the indirect end labeling of genomic fragments, constituting the guanine sequence ladder, must be achieved. The probing strategy, that is, the selection of the "strategic" restriction site in the vicinity of the sequences under study as well as the DNA probe itself, deserves some consideration (see Fig. 3). Under the stringent conditions of UV cross-linking and hybridization described, fragments smaller than 70 nucleotides will not yield a signal. The polyacrylamide gel resolves genomic DNA with single-nucleotide resolution for about 200–250 bp beyond the point where the hybridization signal is picked up (shaded bar in Fig. 3). Thus the restriction site that will become the invariant end should be further than 90 bp away from the region of focus, the resulting fragment on cleavage longer than 400 bp. The

“strategic” restriction endonuclease should cut genomic DNA reliably, be reasonably priced, and not be inhibited by possible methylation of its recognition sequence. Ideally, the probe fragment itself should be a single-copy sequence between 100 and 150 nucleotides long (i.e., short in comparison to the restriction fragment to be labeled, to minimize hybridization to fragments that do not have the invariant end), should be of moderate GC content (35–55% will work), and should be about the strategic restriction site.

Once a suitable restriction site has been selected it is wise to confirm its presence in the genome of the cells used by Southern blotting to rule out the existence of a restriction site polymorphism.

Probe Synthesis

To obtain a suitable hybridization probe as much ^{32}P as possible needs to be incorporated into a relative short stretch of nucleic acid. We favor single-stranded DNA probes over RNA probes produced by transcription with phage polymerases³⁶ because precursor dNTPs of higher specific activity than the corresponding NTPs can be obtained and because DNA polymerases appear to be more efficient in incorporating nucleotides at limiting concentrations. Originally¹⁰ the single-stranded DNA probe was synthesized by primer extension using single-stranded DNA of M13-derived plasmids into which the short probe sequence were cloned. We have described a protocol¹¹ that reproducibly yields high-quality probes following this strategy. A number of disadvantages with this procedure, such as the necessity to clone the probe fragment in both orientations into the M13 vector, the difficulty to confine radioactive labeling to insert sequences only, and the requirement for a gel purification step, have led to the development of a new strategy³⁷ that yields probes of high specific activity from either strand with minimal effort.

Materials

All solutions are prepared with diethyl pyrocarbonate-treated water.

T3/T7 transcription buffer (5 × stock): 200 mM Tris-HCl (pH 8.0), 250 mM NaCl, 40 mM MgCl₂, 10 mM spermidine, 50 mM dithiothreitol (DTT)

NTP mix: ATP, GTP, CTP, and UTP (3.3 mM each); store in aliquots at -20°

DNase I buffer (10 × stock): 500 mM Tris-HCl (pH 7.5), 50 mM MgCl₂, 10 mM DTT

³⁶ K. Zinn and T. Maniatis, *Cell* **45**, 611 (1986).

³⁷ F. Weih, A. F. Stewart, and G. Schütz, *Nucleic Acids Res.* **16**, 1628 (1988).

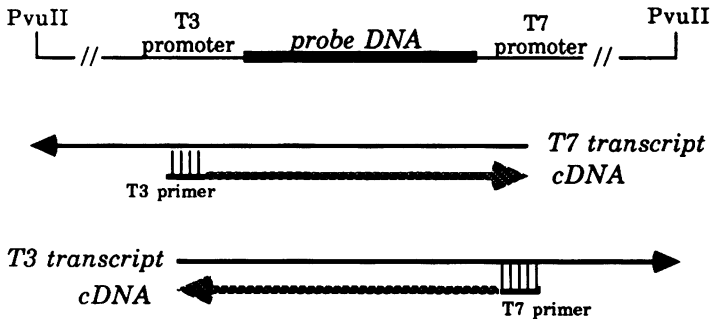


FIG. 4. Probe synthesis by reverse transcription of an RNA template. The upper line displays the *PvuII* fragment from the probe plasmid (probe DNA inserted into the polylinker of pBluescript). The strategy makes use of the symmetric location of two bacteriophage promoters and two "universal" primers that correspond to sequences within the promoters. The procedure is described in detail in the text.

RNase inhibitor: 40 U/ μ l (Stratagene, La Jolla, CA)

RNase-free DNase I: 1 U/ μ l (e.g., Promega, Madison, WI)

RT buffer (5 \times): 250 mM Tris-HCl (pH 8.5; pH 8.3 at 42 $^{\circ}$), 50 mM MgCl₂, 50 mM DTT, 600 mM KCl

dGCT mix: 20 mM dGTP/dCTP/dTTP; store in aliquots at -20 $^{\circ}$

[α -³²P] dATP: 5000-6000 Ci/mmol

AMV reverse transcriptase: Avian myeloblastosis virus reverse transcriptase, 20 U/ μ l (Boehringer Mannheim, Indianapolis, IN)

Procedure

Figure 4 illustrates the principle of the probe synthesis. The small probe fragment is cloned into pBluescribe/pBluescript (Stratagene) or any other vector that contains a multiple cloning site flanked by phage promoters on either side. After digestion of the plasmid with *PvuII* (which cuts on either side of the insert in the vector) a phage polymerase is used to synthesize a large quantity of RNA containing probe and vector sequences up to the *PvuII* site. An aliquot of this RNA then serves as a template for cDNA synthesis. The RNA derived from transcription with T7 polymerase is annealed with the T3 primer and extended by reverse transcriptase in the presence of radioactive dATP to yield a highly labeled cDNA probe specific for one strand of the genomic DNA (see Fig. 4). Correspondingly, a probe for the opposite strand can be derived from the same plasmid construct simply by using the T3 polymerase and T7 primer. The probe synthesis by reverse transcription of an RNA template has a number of advantages over the methods that use M13-derived constructs.

First, by making use of the symmetric structure of vectors carrying two phage promoters, probe DNAs need only be cloned in one orientation. Second, when primers hybridizing close to the insert DNA are employed, the labeling of vector sequences is minimized. Third, large quantities of RNA can be synthesized with the phage polymerases. This RNA can be characterized, accurately quantitated, and then serve as template for multiple rounds of actual probe synthesis. Fourth, once the RNA template has been synthesized, probe preparation is performed in a short time and with minimal exposure to radioactivity. Finally, reverse transcriptase incorporates dNTPs at low concentrations efficiently and thus mostly full-length products are obtained, even under conditions in which only one of the dNTPs is provided as an isotope of the highest specific activity.³⁷

The probe plasmid is cleaved with *PvuII* and the completeness of the digestion verified by agarose gel electrophoresis. Ten micrograms of this DNA (5 pmol of plasmid) is incubated with 20 μ l 5 \times transcription buffer, 10 μ l NTP mix, 2.5 μ l RNase inhibitor (100 U; Stratagene), 5 μ l RNA polymerase (50 U T3 or T7) in a volume of 100 μ l for 15 min at 37°. Thirteen microliters of 10 \times DNase I buffer and 20 μ l of RNase-free DNase I (20 U; Promega) are added and the reaction is incubated for further 15 min at 37° to degrade the DNA template after transcription. The reaction is terminated by phenol and phenol–chloroform (1 : 1) extraction. The following precipitations remove unincorporated nucleotides as well as short DNA fragments. A one-tenth volume of 7.5 M ammonium acetate and 2.5 vol of ethanol are added, mixed, and the reaction chilled for 5 min on ice. After a 5 min spin, the pellet is resuspended in 50 μ l water, precipitated as above, and washed twice with 80% ethanol. After a short period of drying in a speed vacuum concentrator it is dissolved in 20 μ l water. An aliquot can be checked on a RNase-free minigel. The RNA concentration is determined by optical density (OD) measurement of a 1/200 dilution at 260 nm and adjusted to 1 pmol/ μ l. Usually 10–20 pmol transcript is obtained per picomole plasmid DNA. The RNA is stored at –70° and can be used as template for multiple probe synthesis reactions.

The following conditions of reverse transcription attempt to achieve optimal incorporation of a relatively low amount of isotope into largely full-length cDNA. For probe synthesis 250–300 μ Ci [α -³²P] dATP (50 pmol, final concentration 5 μ M) is dried down in a SpeedVac and redissolved in 4 μ l of template RNA (4 pmol). The RNA is denatured by incubation at 70° for 5 min and chilled on ice. To the denatured RNA 2 μ l of the appropriate (T7 or T3) primer (40 pmol; Stratagene), 2 μ l 5 \times RT mix, 0.5 μ l dGTC mix (final concentration, 1 mM for each dNTP), 0.5 μ l RNase inhibitor (Stratagene), and 1 μ l reverse transcriptase are added and the

reaction is incubated for 45 min at 42°. Synthesis is terminated by addition of 1 μ l each of 0.5 M EDTA and 20% (w/v) sodium dodecyl sulfate (SDS). The template RNA is hydrolyzed by addition of 12 μ l 0.4 M NaOH and incubation for 15 min at 70° followed by neutralization with 1 μ l 1 M Tris-HCl (pH 7.5) and 12 μ l 0.4 M HCl. After the addition of 20 μ g carrier tRNA the single-stranded DNA probe is recovered by one selective precipitation, as above. The pellet is dried in a SpeedVac and dissolved in 200 μ l of TE. Incorporation of isotope can be determined with chromatography on polyethylene imine cellulose; the length distribution of the probe DNA should be checked on a denaturing polyacrylamide gel. In general a reaction with a probe fragment (100–150 nucleotides) under the above conditions will result in an incorporation of $1-5 \times 10^8$ dpm.

It is advantageous to insert the probe fragment into the vector such that much of the polylinker is deleted, thus minimizing the contribution of vector sequences to the probe DNA. Should the probe DNA contain a *PvuII* site, *PvuII* cannot be employed to cleave the plasmid. Alternative enzymes should be chosen that produce either blunt or 5' overhang ends (such as *RsaI* or *DdeI*) in order to avoid unspecific initiation of the phage polymerases at 3' overhang ends.

Hybridization

The visualization of the G-specific pattern of a genomic sequence requires that the cDNA probe be able to detect femtogram amounts of specific sequences in the context of a 10^7 -fold excess of unrelated DNA in a highly specific and sensitive hybridization [Fig. 1, step (8)]. The highest sensitivity is needed when working with mammalian genomes, requiring exposure times of at least 1 week. The success of an experiment is thus largely determined by the ratio of specific hybridization to membrane background. The hybridization procedure that was originally introduced by Church and Gilbert¹⁰ is crucial for achieving this goal.

Materials

Na₂HPO₄ (0.5 M), pH 7.2 (solution is 1 M with respect to Na⁺): Dissolve 89 g Na₂HPO₄ · 2H₂O in water, adjust the pH with 85% H₃PO₄, and make up to 1 liter with water

Hybridization buffer: 7% (w/v) SDS [Bio-Rad (Richmond, CA) electrophoresis purity reagent], 1% (w/v) BSA (A7906; Sigma), 1 mM EDTA, 0.25 M Na₂HPO₄ (0.5 M Na⁺), pH 7.2

Wash buffer: 20 mM Na₂HPO₄ (40 mM Na⁺), pH 7.2, 1 mM EDTA, 1% (w/v) SDS; 100 mM NaCl are added for each 5% of reduced GC contents of the probe DNA (from 50%)

Procedure

The most reliable results with a favorable signal-to-noise ratio have been obtained when using a hybridization incubator that contains rotating cylinders made of polypropylene or Plexiglas. The initial experiments in our laboratory were performed with an adapted bacterial incubator and polypropylene measuring cylinders with silicone stoppers. Sophisticated hybridization ovens are now commercially available from a number of companies.

Before each hybridization the cylinders must be cleaned thoroughly with detergent and 10 M NaOH, followed by extensive rinsing with water. The inner surface should not be cleaned mechanically (e.g., with a brush) to avoid scratching the surface. The dry GeneScreen membrane after UV irradiation is wetted by floating on blotting buffer, rolled up, and transferred into the hybridization cylinder. The membrane is manipulated with a thick pipette until it attaches smoothly to the walls without trapping air bubbles. Filling the cylinder with buffer facilitates these manipulations. Multiple layers of membrane do not adversely affect signal or background hybridization. Excess blotting buffer is poured off and 10–20 ml hybridization buffer is added. The cylinder is closed and rotated in the incubator at 65° for at least 30 min. Meanwhile the radioactive probe DNA ($1-5 \times 10^8$ dpm) is added to 10 ml of hybridization buffer and mixed well. After the prehybridization (the length of which is not critical) the hybridization mix is exchanged for the one containing the probe and the cylinder is again rotated at 65° for 16–24 hr. The solution is then poured off and the membrane is rinsed five times with 100 ml wash buffer at 65° while still in the cylinder. The following washes are performed at room temperature in large trays on a platform shaker. Hot wash buffer is poured into a tray and allowed to cool to 65° before the membrane is submerged. Each wash (1 liter of wash buffer) takes about 5 min, during which the buffer slowly cools down. The membrane is then transferred into a second tray again with 1 liter wash buffer at 65°. After eight washes of this kind the membranes are generally clean. The wet membranes are stretched between two layers of Saran wrap and exposed to Kodak (Rochester, NY) XAR film with Du Pont (Wilmington, DE) Cronex Lightning Plus screen at -70°, the DNA-bound side being in contact with the film. After a 20-hr exposure weak signals should be visible that usually allow complete evaluation of the experiment with regard to the quality of the DNA samples and possible footprinted regions. This first exposure also helps to estimate the time finally needed (7–14 days) to obtain publication-quality exposures.

After a satisfactory exposure is obtained, the membrane can be hybridized with the probe specific for the complementary strand. To strip the

old probe, the membrane is submerged in 1 liter 0.2 M NaOH and agitated for 15 min at room temperature. After neutralization with two washes in 100 mM Na₂HPO₄ (pH 7.2), 1 mM EDTA the membrane is ready for rehybridization. The NaOH treatment of the nylon membrane often reduces background as well. With every stripping of the membrane, however, about 10% of the original signal is lost.

Under the given experimental conditions only fragments larger than 70 nucleotides are labeled. We have not determined whether smaller fragments are not retained on the membrane because they are not efficiently cross-linked or because the stringency of hybridization and washing does not allow smaller fragments to hybridize.

The extremely sensitive hybridization technique described above can be used to detect femtogram amounts of specific genomic sequence. However, it also detects equally small amounts of plasmid that may contaminate one or more of the solutions used to prepare the genomic DNA. To avoid such contaminations with, for example, the probe plasmid, we recommend a strict separation of solutions used for *in vivo* footprinting experiments (such as buffers, ethanol, and phenol).

Concluding Remarks

The procedures described in detail have been used with great success in our laboratory as well as in others to obtain information about a large number of different protein-DNA interactions. Among the factors studied using this method were proteins binding to GREs, CREs, CAAT boxes, CACC boxes, G-strings, octamer motifs, and a variety of other sequence modules that have yet to be characterized in more detail. Dimethyl sulfate has proved to be an extremely useful reagent, even though it presents a certain bias to the study of protein binding. Binding to sites that do not contain crucial guanines will go undetected and it is also conceivable that some proteins themselves may be methylated by the agent and dissociate from their binding site. Besides the agent used to footprint a protein *in vivo*, the success of the experiment will be largely determined by the occupancy of a given site. Thus it is of great importance that the cell population to be analyzed be homogeneous with regard to the protein interaction. If only a fraction of the binding sites in the cell pool is occupied by a factor because of asynchrony in cell cycle, variable responsiveness to environmental stimuli, mixed cell types, or simply because the factor does not bind tightly enough *in vivo*, no clear footprint will be obtained. To establish the significance of a specific effect, it is helpful to use control cells that are treated in parallel with the ones under study.

Even though the methods described yield reliable, satisfying results

they should by no means be considered perfect. Without doubt, considerable improvements will be made in the near future as more investigators concentrate their efforts and creativity on further developing the technique. To overcome the need for exposure times in excess of 1 week, linear or polymerase chain reaction (PCR) amplification of genomic sequences has been used to create genomic sequence and footprints.²⁶⁻²⁹ Although exposure times can be reduced using these procedures, we feel that the need to anneal or ligate different oligonucleotides to genomic DNA, with the possibilities of false priming and PCR amplification artifacts, are disadvantages. Direct genomic sequencing will clearly be the method of choice, when less complex genomes (such as *Drosophila* or yeast) are analyzed. We are confident that future improvements will also result in a considerable shortening of exposure times for mammalian genomic footprinting. The most exciting development in this regard will be the substitution of chemiluminescent probes for radioactive labeling.

Acknowledgments

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