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CHROMATIN

# METHODS IN ENZYMOLOGY

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# Section III

## Assays for Chromatin Structure and Function *in Vivo*

# [20] Mapping Chromatin Structure in Yeast

*By* PHILIP D. GREGORY and WOLFRAM HÖRZ

## Introduction

The study of gene expression has become increasingly inseparable from that of chromatin structure. Whereas molecular research previously focused on *cis*-acting DNA elements and the factors that bind to them, the roles that such factors and elements play within a chromatin context will become more and more important. In addition, because the fundamental cellular processes of replication, transcription, recombination, and repair are provided with a nucleosomal DNA substrate, techniques that address the structure of chromatin and that identify perturbations in this matrix are of importance to the study of a wide range of cellular functions and activities.

A unique nucleosomal organization often characterizes the regulatory state of a specific gene. Nuclease digestion experiments originally correlated the appearance of hypersensitivity within a promoter with gene activation, whereas inactive genes maintain a less accessible structure, often with a more regular positioned array of nucleosomes. Recent work has served to underscore the importance of these architectural changes,[1-3] and techniques that facilitate the determination of the underlying chromatin structure can provide important information vital to the understanding of the molecular processes and factors required for proper transcriptional regulation *in vivo*.

To this end, we have successfully employed three basic techniques that provide a high degree of reproducibility to determine the nucleosomal organization of yeast chromatin at various loci. The first employs DNase I with yeast nuclei to determine the presence of positioned nucleosomes or hypersensitive sites within a specific region of chromatin. In addition we provide a micrococcal nuclease-based method that assays for the presence or absence of a nucleosome on a particular stretch of DNA. Although these techniques can provide important structural information, the level of hypersensitivity or the extent to which the chromatin structure is perturbed is hard to assess by such methods. Therefore, we also present a complementary technique that employs restriction enzymes with yeast nuclei to provide quantitative accessibility data. The combination of these methods thus

[1] D. G. Edmondson and S. Y. Roth, *FASEB J.* **10,** 1173 (1996).
[2] P. D. Gregory and W. Hörz, *Eur. Biochem.* **251,** 9 (1998).
[3] A. P. Wolffe, *Nature* **387,** 16 (1997).


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presents an effective diagnostic tool for the study of chromatin structure in yeast.

## Methods

### *Preparation of Yeast Nuclei*

The following protocol for the isolation of yeast nuclei is based on the original method of Wintersberger *et al.*[4] and has been subsequently refined. There are, however, several, more time-consuming methods for the preparation of highly purified nuclei[5,6] and faster procedures for the treatment of nuclei with nucleases in crude lysates.[7–10] The protocol provided here attempts to balance purity and speed and reproducibly provides identical results with DNase I, micrococcal nuclease, and restriction enzyme analyses for which the following protocols are given.

#### *Solutions for Nuclei Preparation*

Preincubation solution: 0.7 *M* 2-mercaptoethanol and 2.8 m*M* EDTA
Sorbitol solution: 1.0 *M* sorbitol
Lysis solution: 1.0 *M* sorbitol and 5 m*M* 2-mercaptoethanol
Ficoll solution: 18% (w/v) Ficoll, 20 m*M* $KH_2PO_4$, pH 6.8, 1 m*M* $MgCl_2$, 0.25 m*M* EGTA, and 0.25 m*M* EDTA
Zymolyase solution: 20 mg Zymolyase 100T (ICN) dissolved in 1 ml water

#### *Procedure*

1. A 1-liter yeast culture is grown to early logarithmic phase (2–4 × $10^7$ cells/ml) and the cells are collected by centrifugation (3000*g*/10 min at room temperature). This is approximately 2–4 OD at 600 nm and should provide approximately 2 g of cells (wet weight) or 0.2 mg of DNA. The measurement of cell density may vary between spectrophotometers, thus counting cells to determine the conversion factor for a particular spectrophotometer is advised. It is important

[4] U. Wintersberger, P. Smith, and K. Letnansky, *Eur. J. Biochem.* **33,** 123 (1973).
[5] J. P. Aris and G. Blobel, *Methods Enzymol.* **194,** 735 (1991).
[6] D. Lohr, *in* Yeast: A Practical Approach (I. Campbell and J. H. Duffus, eds.), p. 125. IRL Press, Oxford, 1988.
[7] M. J. Fedor, N. F. Lue, and R. D. Kornberg, *J. Mol. Biol.* **204,** 109 (1988).
[8] M. W. Hull, G. Thomas, J. M. Huibregtse, and D. R. Engelke, *Methods Cell Biol.* **35,** 383 (1991).
[9] J. M. Huibregtse and D. R. Engelke, *Methods Enzymol.* **194,** 550 (1991).
[10] N. A. Kent, L. E. Bird, and J. Mellor, *Nucleic Acids Res.* **21,** 4653 (1993).

to process a culture immediately after taking cells out of the incubator. Excessive delays or storing the culture first at low temperature may impede the lysis of the cells greatly.

2. Wash cells in ice-cold water and suspend in 50 ml water.
3. Transfer into preweighed 50-ml centrifuge tubes and centrifuge (3000*g*/5 min). Determine wet weight.
4. Add 2 volumes preincubation solution (relative to wet weight of cells) and shake for 30 min at 28°. This step facilitates digestion of the cell wall with Zymolyase. During this time it may be prudent to maintain induction or repression during preincubation and Zymolyase treatment by adding the appropriate inducer or repressor or, for temperature-sensitive strains, adjusting the incubation temperature accordingly.[11] It is also possible to treat cells with Zymolyase in medium supplemented with sorbitol and a reducing agent.
5. Collect by centrifugation (3000*g*/5 min at 5°) and wash in 50 ml 1 *M* sorbitol.
6. Collect again (3000*g*/5 min at 5°) and resuspend in 5 ml lysis solution per 1 g of cells (wet weight).
7. Dilute 20-$\mu$l aliquots 100-fold in water and read optical density at 600 nm, which should be in the range of between 1 and 2.
8. Add 1/50 volume of a freshly prepared Zymolyase solution to the cells.
9. Incubate with gentle agitation at 28°.
10. Measure optical density at 600 nm after 15 and 30 min as in step 7. As a relative measure of lysis, values should drop to 5–20% of the original measurement. Lysis is, however, strain and growth stage dependent, with stationary cells being more difficult to lyse than logarithmically growing cells. We have successfully used nuclei obtained from cells that continued to give 60% of the starting OD value at the end of the Zymolyase treatment. In such cases it is advisable to monitor a constitutively accessible restriction site in chromatin as a control.
11. Centrifuge (2000*g*/5 min at 5°) and wash in 50 ml 1 *M* sorbitol.
12. Centrifuge (3000*g*/10 min at 5°) and resuspend in 7 ml Ficoll solution per 1 g cells (original wet weight). Cells lyse at this stage, but nuclei are stabilized by the Ficoll.
13. Distribute into as many aliquots as desired and centrifuge (30,000*g*/30 min at 5°). Aliquots equivalent to 0.5 or 1 g wet weight cells are suitable for subsequent digestion experiments. Ten-milliliter polypropylene centrifuge tubes are convenient.

[11] A. Schmid, K. D. Fascher, and W. Hörz, *Cell* **71**, 853 (1992).

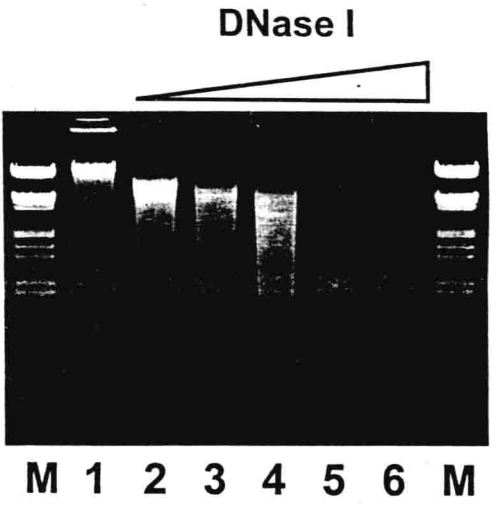



FIG. 1. Monitoring the extent of DNase I digestion of isolated yeast nuclei. Purified yeast nuclei were treated with 0 (lane 1), 0.5 (lane 2), 1 (lane 3), 2 (lane 4), 4 (lane 5), or 8 (lane 6) U/ml DNase I at 37° for 20 min. Marker lanes (M) contain λ DNA digested with *Hin*dIII and *Eco*RI. Suitably digested samples would therefore be in lanes 2–5.

14. Decant supernatant, and freeze the nuclear pellet in liquid nitrogen and store at −70°.

*Chromatin Analysis with DNase I*

The following protocol is designed to assay for histone–DNA interactions and has been used extensively to study inducible *PHO5*,[12] *PHO8*,[13] and *TDH3*[14] promoters. DNase I has also been used extensively to determine the footprints of factors binding to the DNA in chromatin.[8,9] In contrast to the method provided here, these protocols aim to minimize the dissociation of DNA-bound factors by the immediate addition of nuclease following lysis, the use of low salt buffers, and lower digestion temperatures. Furthermore, because these methods assay single-strand cuts rather than the double-strand cuts scored here (see later), the optimum extent of digestion is also lower. An example of the range of DNase I digestion obtained in a digestion series is given in Fig. 1, and an example of a typical result of this procedure

[12] P. D. Gregory, A. Schmid, M. Zavari, L. Lin, S. L. Berger, and W. Hörz, *Mole. Cell* **1,** 495 (1998).
[13] S. Barbaric, K. D. Fascher, and W. Hörz, *Nucleic Acids Res.* **20,** 1031 (1992).
[14] B. Pavlovic and W. Hörz, *Mol. Cell. Biol.* **8,** 5513 (1988).

with respect to the chromatin transition at the *PHO5* promoter is shown in Fig. 2.

*Solutions for DNase I Analysis*

Digestion buffer: 15 m*M* Tris–HCl, pH 7.5, 75 m*M* NaCl, 3 m*M* $MgCl_2$, 0.05 m*M* $CaCl_2$, and 1 m*M* 2-mercaptoethanol

DNase I dilution buffer: 10 m*M* Tris–HCl, pH 7.4, 0.1 mg/ml bovine serum albumin (BSA)

Stop solution: 1.0 *M* Tris–HCl, pH 8.8, and 0.08 *M* EDTA, pH 8.0

Proteinase K solution: 10 mg/ml proteinase K in 10 m*M* Tris–HCl, pH 8.0

Chloroform solution: chloroform/isoamyl alcohol (24:1, v/v)

RNase solution: 5 mg/ml ribonuclease A (DNase-free) dissolved in 5 m*M* Tris–HCl, pH 7.5, and heated for 10 min/100°

*Procedure*

1. Suspend pelleted nuclei from approximately 500-mg cells (wet weight) for one experiment in 3 ml digestion buffer by vortexing.
2. Centrifuge (2000*g*/5 min at 5°) and resuspend in 1.2 ml digestion buffer. Transfer 200-μl aliquots to microfuge tubes.
3. Add DNase I at four different concentrations in the range of 0.5 to 20 U/ml and incubate for 20 min at 37°. Keep one sample on ice and one at 37° without nuclease.
4. Terminate digestion by adding 10 μl stop solution, 5 μl 20% SDS, and 20 μl proteinase K solution. Incubate for 30 min at 37°.
5. Add 1/5 volume 5 *M* $NaClO_4$, 1 volume phenol, vortex, and then 1 volume chloroform solution, vortexing well.
6. Centrifuge for 5 min.
7. Take off supernatant, reextract with 1 volume chloroform solution.
8. Take off supernatant and add 2.5 volumes ethanol to precipitate nucleic acids.
9. Collect by centrifugation and resuspend in 125 μl TE.
10. Add 10 μl DNase-free RNase solution and incubate for 1 hr at 37°.
11. Add 5 μl 5 *M* NaCl and 0.6 volume 2-propanol and centrifuge immediately at room temperature.
12. Wash the pellet with 70% ethanol and dissolve in 80 μl TE.
13. Analyze 5-μl aliquots in 1% agarose gels and stain with ethidium bromide. An example of such a gel is given in Fig. 1.
14. Select appropriate samples and use 20 μl for secondary digestion and indirect end labeling. Optimal extents of digestion may differ according to the chromatin structure of the region of interest and as a product of how distant the region of interest is in relation to

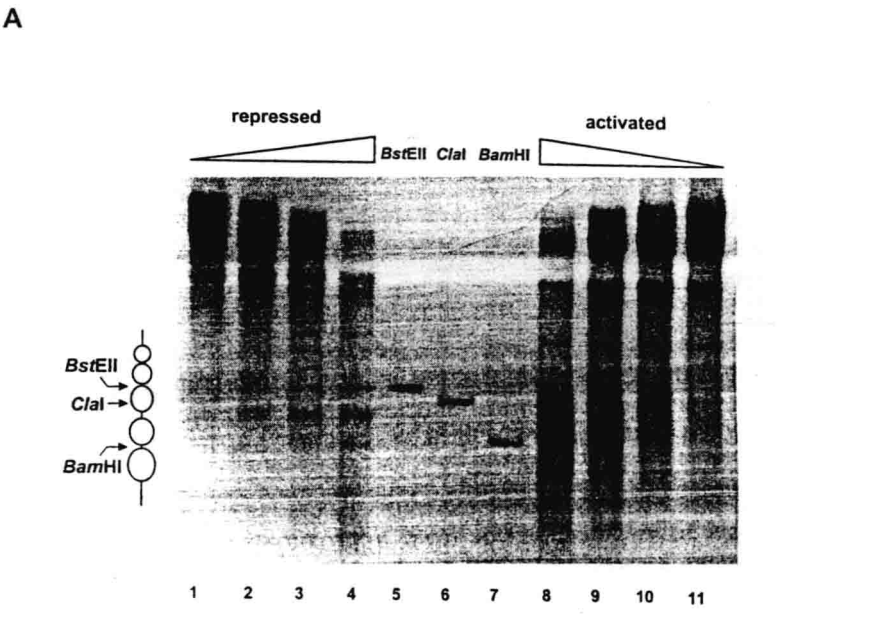



FIG. 2. DNase I analysis of the two chromatin states of the yeast *PHO5* promoter. (A) Nuclei from repressed or activated nuclei were digested with 0.25 (lanes 1 and 11), 0.5 (lanes 2 and 10), 1 (lanes 3 and 9), or 2 (lanes 4 and 8) U/ml DNase I for 20 min at 37°. DNA was isolated, digested with *Apa*I, and analyzed on a 1.5% agarose gel, blotted, and hybridized with probe D.[16] Double digests of purified genomic DNA with *Apa*I/*Bst*EII, *Apa*I/*Cla*I, and *Apa*I/*Bam*HI shown in lanes 5, 6, and 7, respectively, serve as markers. The schematic on the left shows the positions of the restriction sites used for generating the marker fragments together with the derived positions of the nucleosomes. (B) A schematic representation of the chromatin organization of the repressed *PHO5* promoter. The TATA box (T) and the positions of the Pho4-binding sites UASp1 and UASp2 are shown. Nucleosomes removed on activation are shown as open circles. Also shown are probe D and the location of the *Apa*I site used for indirect end labeling.

the secondary digest and probe position. In general, a range of digests are taken for secondary digestion, which should show neither a high proportion of mononucleosome fragments nor no apparent change on digestion. For an example of a suitable extent of digestion, see Fig. 1, lanes 2–5.

15. Results of a typical analysis are given in Fig. 2.

*Mononucleosome Analysis with Micrococcal Nuclease*

Micrococcal nuclease demonstrates strong DNA sequence specificity and shows preference for DNA in the linker region between positioned nucleosomes.[15] The method described here utilizes the latter to reduce the chromatin into mono-, di-, tri-, and tetranucleosomal DNA fragments. The DNA is then resolved on an agarose gel and is analyzed by Southern blotting and hybridization to short DNA probes (see the schematic in Fig. 3A). The assay asks whether there is a nucleosome on a given stretch of DNA. Because the technique does not utilize indirect end labeling, the assay does not provide information relating to nucleosome position. One advantage of this method, however, is that by direct comparisons between the results of the same blot probed with several different DNA fragments it is possible to effectively "walk" across a region of chromatin determining the presence or absence of nucleosomes. To exemplify this technique, it has been used to study the chromatin of the *PHO5* promoter[11,16] (see Fig. 3B) and the *Gal1-10* promoter.[17]

*Solutions*

Digestion buffer: 15 m*M* Tris–HCl, pH 8.0, 50 m*M* NaCl, 1.4 m*M* $CaCl_2$, 0.2 m*M* EDTA, 0.2 m*M* EGTA, and 5 m*M* 2-mercaptoethanol

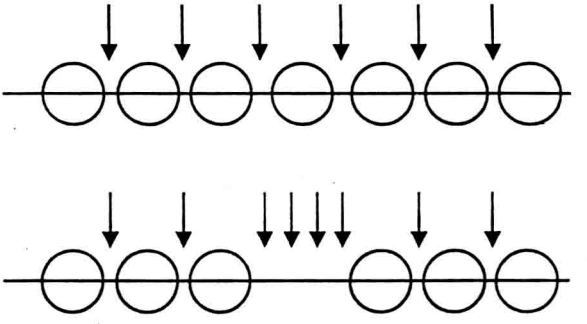
Nuclease dilution buffer: 10 m*M* Tris–HCl, pH 7.4, and 0.1 mg/ml BSA

*Procedure*

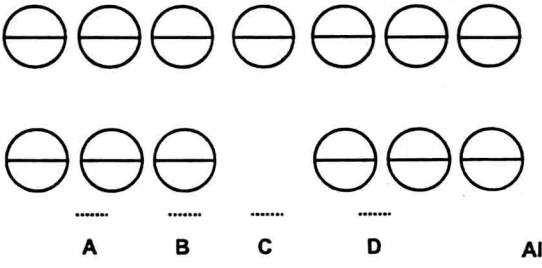
1. Suspend nuclei from approximately 500-mg cells (wet weight) in 3 ml digestion buffer by vortexing.
2. Centrifuge (2000*g*/5 min at 4°), resuspend in 1.2 ml digestion buffer, and transfer 200-$\mu$l aliquots to microfuge tubes.
3. Add micrococcal nuclease [Boehringer Mannheim (also listed as nuclease S7)] at four different concentrations in the range of 5 to 100 U/ml and incubate for 20 min at 37°. The extent of digestion is

[15] W. Hörz and W. Altenburger, *Nucleic Acids Res.* **9,** 2643 (1981).
[16] A. Almer, H. Rudolph, A. Hinnen, and W. Hörz, *EMBO J.* **5,** 2689 (1986).
[17] M. J. Fedor and R. D. Kornberg, *Mol. Cell. Biol.* **9,** 1721 (1989).

**A** **1. Micrococcal nuclease digestion**




**2. Gel electrophoresis, Southern transfer, hybridization, and autoradiography**




**3. Mononucleosome signal**

| Probe | Repressed | Active |
|---|---|---|
| A | + | + |
| B | + | + |
| C | + | - |
| D | + | + |

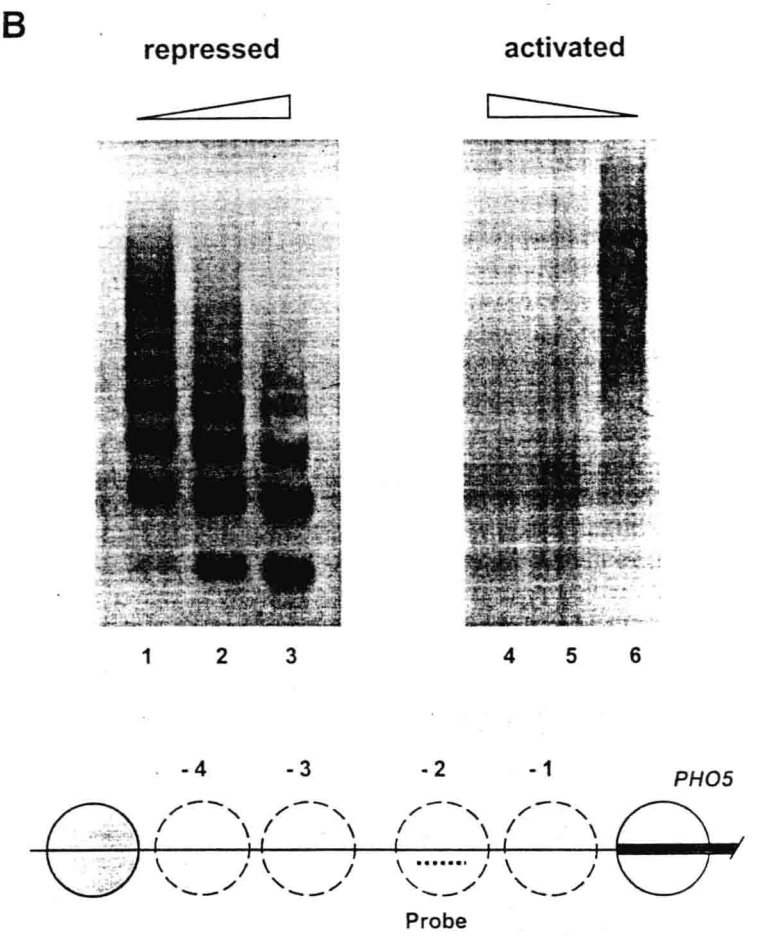



FIG. 3. Assaying for the presence or absence of nucleosomes. (A) A schematic of the protocol. (B) Nuclei isolated from repressed or activated cells were treated with 32 (lanes 1 and 6), 64 (lanes 2 and 5), and 128 (lanes 3 and 4) U/ml micrococcal nuclease. DNA was isolated, separated on a 2% agarose gel, blotted, and hybridized to a nucleosome -2 specific probe. The schematic underneath depicts the chromatin organization of the *PHO5* promoter and the position of the probe employed. Nucleosomes disrupted under activating conditions are shown as dashed circles.

monitored by ethidium bromide-stained gels. Select samples that have mostly mononucleosomes and also samples that still have di-, tri-, and tetranucleosomal DNA.

4. Terminate digestion by adding 10 $\mu$l stop solution, 5 $\mu$l 20% SDS, and 20 $\mu$l proteinase K solution. Incubate for 30 min at 37°.

5. Follow steps 5 to 14 of the protocol given for DNase I digestion.
6. Analyze by agarose gel electrophoresis (2% is appropriate), Southern transfer without secondary digestion, and hybridization. A typical result is shown in Fig. 3B.

*Restriction Enzyme Accessibility Assay*

A limitation of the use of nonspecific nucleases in the study of chromatin structure is the inherent difficulty in determining the extent to which a chromatin modulation has occurred. The presence of a nucleosome is an effective barrier to restriction enzymes and prevents the digestion of sites within the underlying DNA, whereas transcription factors, at least under the conditions employed, do not. A restriction enzyme analysis provides a relatively easy and reliable method of quantifying the accessibility of nucleosomal DNA and is therefore an excellent complementary technique to the more standard DNase I analysis. This analysis can provide information on the boundaries of the observed transition, the nature of the transition and resultant structure, and the question of what proportion of the cell population undergoes this transition. The strategy employed is shown schematically with a typical result of the analysis in Fig. 4. This technique has also been used to determine the influence of histone acetylation at the *PHO5* promoter.[12]

It should be noted that to demonstrate protection conclusively it is necessary to show that at least one other site was cut by the enzyme in a particular digest, as the default state for the majority of sites in chromatin will be "protected." Intranucleosomal sites are typically 5–10% accessible, whereas sites located within hypersensitive regions demonstrate 80–95% cleavage, e.g., the *PHO5*[16] or *PHO8*[13] promoter.

*Solutions for Restriction Enzyme Analysis*

Digestion buffer: 10 m*M* Tris–HCl, pH 7.4, 50 m*M* NaCl, 10 m*M* $MgCl_2$, 0.5 m*M* spermidine, 0.15 m*M* spermine, 0.2 m*M* EDTA, 0.2 m*M* EGTA, and 5 m*M* 2-mercaptoethanol

*Procedure*

1. Suspend pelleted nuclei in digestion buffer by vortexing.
2. Centrifuge (2000*g*/5 min at 5°) and resuspend in digestion buffer. Nuclei from approximately 50-mg cells (wet weight) are used for one experiment and are suspended in 200 $\mu$l. Transfer to microfuge tubes. The buffer employed balances the need to preserve the chromatin structure while facilitating enzyme activity. The buffer therefore contains 50 m*M* salt, which appears to be sufficient for most enzymes

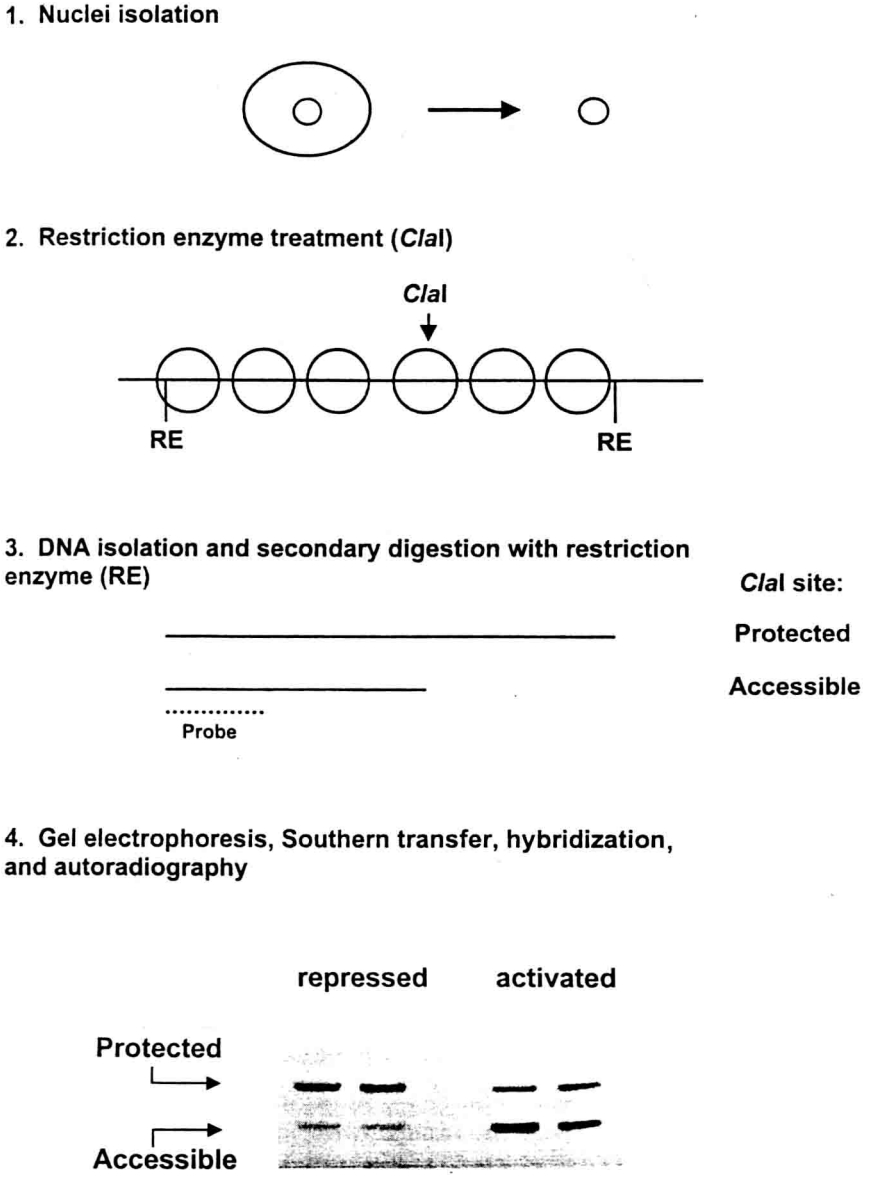



FIG. 4. Restriction site accessibility of the *PHO5* promoter. Nuclei isolated from repressed and activated strains were treated with either 50 (left) or 200 (right) u of *Cla*I and incubated for 30 min at 37°. In order to monitor the extent of cleavage, DNA was isolated, cleaved with *Hae*III, analyzed on a 1.5% agarose gel, blotted, and hybridized with probe D.[16] A schematic representation of the method is provided at the top.

in the chromatin digestion, and the polyamines, spermidine and spermine, which serve to stimulate restriction enzyme activity and (usually) suppress nonspecific cellular nucleases.

3. Add restriction nuclease at two different concentrations (range between 150 and 1500 U/ml). Incubate at 37° for 30 min. The two concentrations of enzyme differing by a factor of 3–4 should provide approximately identical levels of cleavage. This demonstrates that the amount of enzyme was not limiting. Therefore, all accessible sites can be assumed to be cut within the time course of the experiment. It is also advisable to include a 0 and 37° control to monitor the extent to which endogenous nucleases were active during the incubation. In this respect it is preferable if the longer (protected) and shorter (accessible) fragments generated by the analysis are of an approximately similar size and thus presumably equally susceptible to degradation by endogenous nucleases.
4. Terminate digestion as described for DNase I except raise the EDTA concentration to 12 m*M*.
5. Follow steps 5 to 14 of the protocol given for DNase I analysis.
6. Use 10 $\mu$l of the DNA from restriction nuclease-digested nuclei for secondary digestion with the appropriate restriction enzyme.
7. Analyze by Southern transfer and hybridization (Fig. 4).
8. Accessibility can be quantified by determining the ratio of the amount of each fragment per lane by phosphoimaging analysis or densitometry.

### Acknowledgments

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## [21] Assays for Nucleosome Positioning in Yeast

*By* MICHAEL P. RYAN, GRACE A. STAFFORD, LIUNING YU, KELLIE B. CUMMINGS, and RANDALL H. MORSE

### Introduction

The yeast *Saccharomyces cerevisiae* is an excellent eukaryotic model system to investigate how chromatin functions in biological processes such




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as transcription, replication, and silencing. To understand the relationship between chromatin structure and function, it is critical to define where nucleosomes, the repeating unit of chromatin, are positioned along a DNA sequence. Each nucleosome contains 147 bp of DNA wrapped $1\frac{3}{4}$ turns around a cylindrical-shaped histone core consisting of two molecules each of histones H2A, H2B, H3 and H4 (reviewed in Wolffe[1]). In higher eukaryotes, histones H1 and H5 bind to DNA entering and exiting the nucleosome and promote higher order folding of chromatin. Although a homolog of histone H1 has been identified in yeast, genetic studies suggest that it plays a minor role with respect to chromatin structure and function.[2]

Positions of nucleosomes are defined translationally and rotationally.[3,4] Translational positioning specifies where the histone core begins and ends along the DNA sequence. Rotational positioning refers to the orientation of the DNA helix relative to histone core, e.g., whether the minor groove faces inward to the core or outward. Several techniques have been developed to map both the translational and the rotational positioning of nucleosomes. In general, these techniques utilize enzymes or chemicals that cleave nucleosomal and nonnucleosomal DNA differentially. Differential cleavage is largely due to the wrapping of DNA around the nucleosome core, which makes DNA in chromatin less accessible to cutting.

When mapping the structure of chromatin in yeast, the position of a nucleosome is determined from a population of sequences. Within this population, a given region of DNA may contain nucleosomes that are randomly or well positioned. Well positioned implies that the nucleosome occupies a particular sequence in the majority of the population being examined. Well-positioned nucleosomes have been characterized in the promoter regions of a number of yeast RNA polymerase II-dependent genes in their inactive state, including the *PHO5, GAL1*, and *GAL10* genes, and in yeast minichromosomes.[5–8] In many cases, the mechanism for positioning is unclear. Experimental evidence indicates that nucleosome positioning can be directed by the DNA sequence and by DNA-binding proteins that position nucleosomes (e.g., the $\alpha 2$ repressor protein[7]) or gener-

[1] A. P. Wolffe, "Chromatin: Structure and Function." Academic Press, New York, 1995.
[2] H.-G. Patterton, C. C. Landel, D. Landsman, C. L. Peterson, and R. T. Simpson, *J. Biol. Chem.* **273,** 7268 (1998).
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[4] F. Thoma, *Biochem. Biophys. Acta* **1130,** 1 (1992).
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[6] J. Svaren and W. Hörz, *TIBS* **22,** 93 (1997).
[7] S. Y. Roth, A. Dean, and R. T. Simpson, *Mol. Cell. Biol.* **10,** 2247 (1990).
[8] R. H. Morse, *Science* **262,** 1563 (1993).