

using primer extension methodology. If the minichromosome is to serve as a valid model for the genomic situation, chromatin structure of the amplified, isolated gene and the genomic, native gene should be identical.

More direct structural analysis of the regulated gene chromatin in different functional states is possible with isolation of minichromosomes. For example, we have found distinctive chromatin architectures for active and repressed **a**-cell specific genes and for the silent mating type loci (C. L. Woodcock and R. T. Simpson, unpublished observations), using electron microscopy of minichromosomes. The possibility of three-dimensional reconstructions of either total minichromosomes or the structured domains by negative staining, or possibly by cryoelectron microscopy, so successful with the yeast SWI/SNF chromatin remodeling complex,¹⁰ is enticing.

Functional analysis leading to mechanistic insight concerning gene regulation has been more difficult, even with isolated minichromosomes. One group has shown that minichromosomes (isolated by a different method) retain RNA polymerase II on an active gene and can generate run-on transcripts.¹¹ The goal of using isolated minichromosome transcription templates with an *in vitro* transcription system remains elusive.

Acknowledgment

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¹⁰ C. L. Smith, R. Horowitz-Scherer, J. F. Flanagan, C. L. Woodcock, and C. L. Peterson, *Nat. Struct. Biol.* **10**, 141 (2003).

¹¹ C. H. Shen, B. P. Leblanc, J. A. Alfieri, and D. J. Clark. *Mol. Cell. Biol.* **21**, 534 (2001).

[11] Purification of Defined Chromosomal Domains

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Introduction

A mechanistic understanding of transcriptional activation ultimately depends on its reconstitution *in vitro*. Genetic and biochemical evidence has shown that the activation of transcription involves the alteration of chromosomal proteins and chromatin structure. The alterations include

covalent modifications of histones, exposure of promoter DNA, and changes in protein composition. Mechanistic studies to date have been limited to the use of artificial chromatin, reconstituted *in vitro*. It is unclear how these templates relate to the chromatin of transcriptionally active or repressed genes *in vivo*. The isolation of chromatin assembled *in vivo* may bridge the gap between natural and artificial chromosomal material. Here we describe a method for the isolation of genes from *Saccharomyces cerevisiae* in the form of chromatin in different transcriptional states. The method is an extension of the work of Gartenberg and co-workers, who employed site-specific recombination *in vivo* and differential centrifugation to separate selected chromosomal regions from bulk chromatin.¹ We have used affinity chromatography to purify the selected chromosomal regions to near homogeneity.

Genetic Manipulations

The isolation of a defined chromatin domain requires its excision from the chromosomal locus and can be facilitated by the inclusion of a DNA sequence to serve as a binding site for affinity purification (Fig. 1). The requirement for excision may be met by flanking the genomic region of interest with recognition sites (RS elements; Fig. 1) for the R-recombinase of *Zygosaccharomyces rouxii*. The recombinase excises the region between the two RS sites, releasing it in circular form. For affinity purification, we have incorporated a cluster of LexA-binding sites (L; Fig. 1) adjacent to an RS site, such that it is included in the excised chromatin domain. The cluster serves as a target sequence for a recombinant adapter protein, which contains the entire coding sequence for LexA from *Escherichia coli* at its N terminus, followed by a simian virus 40 (SV-40) nuclear localization signal, a linker region bearing the Garnier–Robson helix 6 from rat plectin,² and a C-terminal TAP tag.³

Standard molecular cloning techniques⁴ and published procedures for genomic manipulation of yeast⁵ are employed as follows.

¹ A. Ansari, T. H. Cheng, and M. R. Gartenberg, *Methods* **17**, 104 (1999).

² F. A. Steinbock and G. Wiche, *Biol. Chem.* **380**, 151 (1999).

³ G. Rigaut, A. Shevchenko, B. Rutz, M. Wilm, M. Mann, and B. Seraphin, *Nat. Biotechnol.* **17**, 1030 (1998).

⁴ J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

⁵ R. Rothstein, *Methods Enzymol.* **194**, 281 (1991).

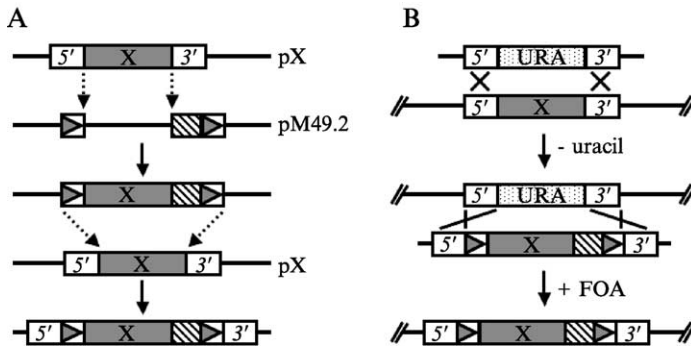


FIG. 2. Construction of a genetically modified locus in yeast. (A) Cloning strategy to modify a locus of interest. pX and pM49.2, cloning vectors. (B) Incorporation of the modified locus in the genome. A two-step replacement strategy using *URA3* as a counterselectable marker is employed.

3. The chromosomal wild-type copy of the gene is exchanged with the modified locus by a two-step gene replacement approach, based on homologous recombination, employing positive and negative selection for the *URA3* marker gene (Fig. 2B).

4. Yeast strains are transformed with two plasmids: plasmid pB3 contains the coding sequence for the R-recombinase under the control of the inducible *GALI* promoter; plasmid pJSS3.1 expresses the recombinant adapter protein under the control of a constitutive glyceraldehyde phosphate dehydrogenase (GPD) promoter.

Plasmids and Yeast Strains

Plasmid pM49.2 is a derivative of pABX22, a kind gift of M. Gartenberg (the University of Medicine and Dentistry of New Jersey, Piscataway), and has been modified by addition of a LexA-binding cluster juxtaposed to an RS element. The LexA-binding cluster is a concatemer of three copies of the LexA operator from the *ColEI* gene of *E. coli* with the sequence GCTGTATATAAAACCAGTGGTTATATGTACAGTA. The creation of single restriction sites flanking the RS and LexA-RS elements in pM49.2 simplifies cloning strategies for the modification of many genomic loci.

Plasmid pB2 was obtained by flanking the *LEU2* gene in pRS415-RecR¹ with RS elements. Yeast cells transformed with this plasmid and grown in the absence of leucine eliminate clones that express the

recombinase prematurely (i.e., before induction with galactose). Plasmid pB3 was derived by digesting plasmid pB2 with *BaeI* and religating, thereby deleting a potential LexA-binding site.

To generate plasmid pJSS3.1, full-length LexA, plectin spacer, and TAP tag were amplified by three separate PCRs, using *E. coli* genomic DNA, rat genomic DNA, and plasmid pBS1479⁶ as templates, respectively. Primers were designed to introduce novel restriction sites in-frame for subsequent ligation into the complete construct. The final three-part ligation product was subcloned into the *E. coli*/yeast centromeric shuttle vector p416-GPD⁷ (Funk, ATCC 87360).

Yeast strains used must be deficient in *URA3* and *LEU2* expression to allow genomic manipulation and to stably harbor pB3 and pJSS3, containing *LEU2* and *URA3* markers, respectively.

Cell Growth and Cell Disruption

Yeast strains transformed with plasmids pB3 and pJSS3 are grown at 30° in Hartwell's synthetic medium lacking leucine and uracil, containing 2% (w/v) D-glucose (SCD) or 2% (w/v) D-raffinose (SCR) as a carbon source. Starter cultures are routinely grown in SCD, because glucose repression of the *GALI* promoter prevents premature excision of chromatin circles from the endogenous locus due to leaky recombinase expression. Induction of the recombinase by the addition of galactose releases about 75% of the chromatin from the endogenous locus.

Because the intactness of genomic DNA is a prerequisite for its removal in the subsequent differential centrifugation steps (see below), cells are lysed in liquid nitrogen, which limits fragmentation of chromosomes. Efficiency of lysis is between 60 and 70% in the following procedure.

1. Cells from a 200-ml culture grown overnight in SCD are collected by centrifugation (5 min, 2200g at room temperature), washed twice with water, and used to inoculate 12 liters of SCR in a fermentor. Typical starting densities for the 12-liter cultures are between 3.5×10^6 and 5×10^6 cells/ml. Cells are grown overnight, stirred at 500 rpm with mild aeration.

2. Recombination is induced at cell densities between 0.5×10^8 and 1×10^8 cells/ml, by adding 600 ml of 40% (w/v) D-galactose. Cells are grown for an additional 1 h and 30 min.

⁶ O. Puig, F. Caspary, G. Rigaut, B. Rutz, E. Bouveret, E. Bragado-Nilsson, M. Wilm, and B. Seraphin, *Methods* **24**, 218 (2001).

⁷ D. Mumberg and M. Funk, *Gene* **156**, 119 (1995).

3. Cells are harvested by centrifugation (10 min, 9000g at 4°), yielding a wet weight between 30 and 40 g. After washing twice with water, cells are pelleted in sealed 20-ml syringes by centrifugation (5 min, 3000g at 4°). Syringes are unsealed by cutting with a razor blade, supernatants are decanted, and cells are extruded into liquid nitrogen.⁸ Cells can be stored at -80°.

4. Frozen cells are ground in a liquid nitrogen-dry ice mixture in a 1-liter stainless steel container, using a commercial blender (Waring, 7011HS). The blender is run for 10 min, alternating between low and high blending speed at 30-s intervals. Evaporated nitrogen is replaced while grinding at low blending speed. Occasional tapping with a spatula against the outside of the container prevents ground cells from sticking in a layer to the inside wall of the blender. The fine powder of ground yeast can be stored at -80°.

Extraction and Differential Centrifugation

A differential centrifugation protocol¹ allows separation of the circular chromatin domains released by site specific recombination from bulk genomic DNA. Cell debris and bulk genomic DNA are removed in an initial low-speed spin, leaving chromatin circles in the supernatant, which can be sedimented in a second spin at higher centrifugal force. Centrifugal forces and centrifugation times must be adjusted according to the chromatin domain under study, and should be determined in pilot experiments. About half of the starting circles can be recovered in the second pellet, in the case of circles containing 2.2 kb of DNA. For smaller circles, with a size of 0.75 kb, a recovery of 65% is obtained.

The following steps are performed at 4°.

1. Ground cells are extracted by the addition of buffer A (5 ml/g of cells), containing protease inhibitors, and stirring for 15 min.
2. The crude extract is clarified by centrifugation at 72,700g for 1 h.
3. Chromatin circles present in the supernatant are sedimented by centrifugation at 371,000g for 2 h (for circles bearing 2.2 kb of DNA), or 3 h (for circles bearing 0.75 kb of DNA) and resuspended in 1/10 of the original volume of buffer B, containing protease inhibitors. The protein concentration of the resuspended circles is between 5 and 10 mg/ml. The resuspended chromatin circles can be stored at -80°.

⁸ M. C. Schultz, S. Y. Choe, and R. H. Reeder, *Proc. Natl. Acad. Sci. USA* **88**, 1004 (1991).

Reagents

Buffer A: 0.2 M potassium acetate, 2 mM EDTA, 10% (v/v) glycerol, 125 μ M spermidine, 50 μ M spermine, 5 mM 2-mercaptoethanol, and 25 mM HEPES-KOH (pH 7.4)

Buffer B: 0.1 M potassium acetate, 1 mM EDTA, 10% (v/v) glycerol, 125 μ M spermidine, 50 μ M spermine, 5 mM 2-mercaptoethanol, and 25 mM HEPES-KOH (pH 7.4)

Protease inhibitors (100 \times concentrate): benzamidine (33 mg/ml), PMSF (17 mg/ml), pepstatin A (137 μ g/ml), leupeptin (28.4 μ g/ml) in ethanol

Affinity Purification

Differential centrifugation enriches not only for chromatin circles, but also for ribosomes, minichromosomes (plasmids pB3, pJSS3.1, and 2 μ circle), and high molecular weight protein complexes. The resuspended pellet is a crude mixture of proteins and nucleic acids. The presence of nucleases and ATP-dependent chromatin-remodeling activities at this stage of the purification makes the material unsuitable for use in biochemical assays.

The recombinant LexA adapter molecule expressed in the yeast cells permits tandem affinity purification (TAP)⁶ for the isolation of adapter-bound chromatin domains. Most contaminating ribosomal material is removed in a first affinity step, involving interaction between the protein A component (Prot A; Fig. 1) of the adapter and IgG-agarose beads. Because the bound material is released from the beads with recombinant His-tagged tobacco etch virus (TEV) protease, the protease itself contaminates the affinity eluate. If the stringency of washes in the subsequent calmodulin-binding peptide (CBP; Fig. 1)/calmodulin-agarose affinity purification does not suffice to remove all traces of the protease, the His-tagged enzyme can be eliminated by an additional incubation with Talon beads.

We have found that stringent wash conditions, such as 0.4 M potassium acetate or 0.1% Nonidet P-40 (NP-40), do not adversely affect the structural or topological integrity of the isolated chromatin domains (see later). The efficiency of the tandem affinity purification is dependent on the size of the chromatin circles: 22% of the input can be recovered for 2.2-kb circles, whereas 45% recovery is obtained for 0.75-kb circles.

Affinity chromatography is performed at 4 $^{\circ}$, under gravity flow, with insulin added at 0.1 mg/ml to all buffers except buffer C, as follows.

1. IgG–agarose beads (1/20 of the applied volume) are equilibrated with 20 bead volumes of buffer B in 20-ml Econo-Pac plastic columns (Bio-Rad, Hercules, CA), and incubated overnight in 2 bead volumes of the same buffer.

2. The resuspended pellet from differential centrifugation is diluted with an equal volume of buffer B containing protease inhibitors, resulting in protein concentrations between 2.5 and 5 mg/ml. The mixture is centrifuged at 21,200g for 5 min and the clear supernatant is applied to the IgG–agarose beads. The top and bottom of the columns are sealed and the contents are mixed by slow rotation for 2 h.

3. The flow-through is collected and the beads are washed with 40 bead volumes of buffer B without glycerol, containing 0.4 M potassium acetate, and subsequently with 40 bead volumes of buffer B without glycerol, containing 50 mM potassium acetate and 0.1% NP-40.

4. Chromatin circles are released by proteolytic cleavage for 1 h with recombinant TEV protease (80 ng/ml) in 1.2 bead volumes of buffer C, with occasional mixing. To wash residual chromatin circles off the column, 4 bead volumes of buffer B is added sequentially. For freezing, the glycerol concentration is adjusted to 10% and the eluates are stored at -80° .

5. Calmodulin beads (1/36 of the applied volume) are equilibrated with 20 bead volumes of buffer D in 20-ml Econo-Pac plastic columns and incubated overnight in 2 bead volumes of the same buffer.

6. The magnesium and calcium ion concentrations in the IgG–agarose eluates are adjusted by addition of 1 M magnesium acetate (2 μ l/ml) and 1 M calcium chloride (3 μ l/ml). The mixture is centrifuged at 21,200g for 5 min and the clear supernatant is applied to the calmodulin beads. The top and bottom of the columns are sealed and the contents are mixed by slow rotation for 1 h.

7. The flow-through is collected and the beads are washed with 80 bead volumes of buffer D containing 0.3 M potassium acetate and 0.1% NP-40.

8. Chromatin circles are eluted with buffer E, in six fractions of 2 bead volumes each. Chromatin circles are recovered in the first three fractions. For freezing, the glycerol concentration is adjusted to 10% and the eluates are stored at -80° .

Reagents

IgG–agarose beads (Sigma, St. Louis, MO)

Calmodulin affinity resin (Stratagene, La Jolla, CA)

Talon metal affinity resin (BD Biosciences Clontech, Palo Alto, CA)

TEV protease (GIBCO-BRL, Gaithersburg, MD)

Buffer C: 0.2 M potassium acetate, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 25 mM HEPES-KOH (pH 7.4)

Buffer D: 0.1 M potassium acetate, 1 mM imidazole, 2 mM calcium chloride, 1 mM magnesium acetate, 125 μ M spermidine, 50 μ M spermine, 10 mM 2-mercaptoethanol, and 25 mM HEPES-KOH (pH 7.4)

Buffer E: 0.2 M potassium acetate, 2 mM EGTA, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 25 mM HEPES-KOH (pH 7.4)

Comments

The combination of differential centrifugation and tandem affinity purification permits the separation of a specific chromosomal locus from virtually all nucleic acids and most of the proteins present in the cell. After purification of *PHO5* chromatin as described above, *PHO5* DNA is present in 20- to 30-fold excess over all other chromosomal DNA. The only nucleic acid detectable in the final eluate by agarose gel electrophoresis and ethidium bromide staining is *PHO5* circle DNA. The purified material is free from chromatin-remodeling activities, nucleases, proteases, and topoisomerases. Using this approach, we have found that different transcriptional states of *PHO5* chromatin are stable *in vitro*, conserving important structural and topological features of the *PHO5* locus *in vivo*.⁹

Even with the procedures described above, the goal of chromatin purification has not been fully achieved. Purification of a single-copy gene entails its separation from a 10,000-fold excess of genomic DNA and a 1 million-fold excess of contaminating proteins. Our procedures yield 0.1 to 0.3 pmol of *PHO5* DNA from 10^{12} cells (corresponding to 30–40 g wet weight). This quantity is at the lower end of sensitivity of mass spectrometry. The total amount of contaminating proteins carried through the purification is still 10-fold above the amount of histone associated with the isolated DNA circle. Additional steps for enrichment of isolated circles are under investigation.

⁹ H. Boeger, J. Griesenbeck, J. S. Strattan, and R. D. Kornberg, *Mol. Cell* **11**, 1587 (2003).