Analysis of Chromatin Transitions by Real-Time Quantitative PCR

Joachim Griesenbeck,[1] J Seth Strattan,[1] Hinrich Boeger,[1] Amy Tam,[2] David Batey,[2] and Roger Kornberg,[1] [1]Stanford University, Palo Alto, CA 94305 USA, and [2]Bio-Rad Laboratories, Inc., 2000 Alfred Nobel Drive, Hercules, CA 94547 USA

Introduction

In the eukaryotic nucleus, DNA is compacted into chromatin, a highly structured nucleoprotein complex whose basic repeated unit, the nucleosome, consists of ~150 bp of DNA wrapped around a histone protein octamer. The nucleosome efficiently packages large genomes but can impede access to DNA by important regulatory molecules. Transcriptional initiation, for example, requires that promoter regions be available for the assembly of the basic transcription machinery. Changes in DNA accessibility, such as those required for transcription, are referred to as chromatin transitions.

We describe a method to monitor chromatin transitions at the *PHO5* promoter, which undergoes profound changes in its chromatin structure upon transcriptional activation (Boeger et al. 2003). During activation, the *PHO5* promoter region that is protected by nucleosomes under repressed conditions becomes exposed. A hallmark of this chromatin transition is increased accessibility of a *Cla*I restriction site within the region. The assay we describe tests the accessibility of the yeast *PHO5* promoter region to cleavage by the *Cla*I restriction endonuclease, which can be quantitated using real-time quantitative PCR (qPCR) in cells repressed or activated for *PHO5* expression.

An overview of the workflow for this assay is presented in Figure 1. Briefly, yeast nuclei are isolated from cells grown under conditions that repress or activate *PHO5* gene expression, and the intact nuclei are then treated with *Cla*I. After isolation of the genomic DNA, the extent of *Cla*I digestion is commonly determined by Southern blot analysis (Boeger et al. 2003); here, however, real-time qPCR is used to quantitate the amount of DNA inaccessible to (not cleaved by) *Cla*I. The chromatin transition assay described here showed increased cleavage (accessibility to *Cla*I) under activating conditions, and this result is consistent with a model that nucleosomes are removed during transcriptional activation of the *PHO5* promoter (Almer et al. 1986).

Methods

Restriction Endonuclease Digestion of Yeast Nuclei

Cultures of the yeast *Saccharomyces cerevisiae* were grown under conditions that repress or activate *PHO5* transcription, and nuclei were prepared as described by Almer et al. (1986). The nuclei (0.1 fmol) were then incubated in the absence or presence of *Cla*I restriction endonuclease for 30 min at 37°C in 30 µl of a buffer containing 25 mM HEPES-KOH, pH 7.4, 0.1 M potassium acetate, 7.5 mM magnesium sulfate, 5% PEG 6000, 5 mM β-mercaptoethanol, and 0.1 mg/ml insulin. The reaction was stopped upon addition of 30 µl of a buffer containing 500 mM sodium chloride, 20 mM EDTA, 1% SDS, 50 mM Tris-HCl, pH 8.0, and 1.4 U of proteinase K (Sigma-Aldrich) and incubation for 30 min at 37°C. After phenol-chloroform extraction, DNA was precipitated with ethanol and fragmented by digestion with *Kpn*I in a reaction volume of 50 µl. After complete *Kpn*I digestion, 4 µl of a 1:200 dilution in TE buffer was used for qPCR reactions. Another aliquot of the *Kpn*I-digested DNA that had not been subjected to *Cla*I digestion was diluted 1:100 in TE buffer, and a 2-fold dilution series was used to generate a standard curve.

qPCR for Restriction Endonuclease Accessibility Assays

Triplicate reactions were performed for each sample in a final volume of 40 µl of 1x DyNAmo SYBR Green qPCR kit (Finnzymes) using the DNA Engine Opticon® system. Components for qPCR reactions are listed in Table 1. Primers were designed to amplify two distinct regions of the yeast *PHO5* promoter: Primers P122 (5'-TTTCGCATAGAACGCAACTG-3') and P123 (5'-ATGCCTTGCCAAGTAAGGTG-3') amplify a 206 bp fragment that has a *Cla*I restriction site; primers P128 (5'-TCATCTTATGTGCGCTGCTT-3') and P129

Table 1. qPCR reaction components.

Component	Stock Concentration	Volume (µl)	Final Concentration
DNA template		4.0	
Forward primer	10 µM	0.8	200 nM
Reverse primer	10 µM	0.8	200 nM
DyNAmo SYBR Green qPCR kit	2x	20.0	1x
Water		14.4	
Total		40.0	

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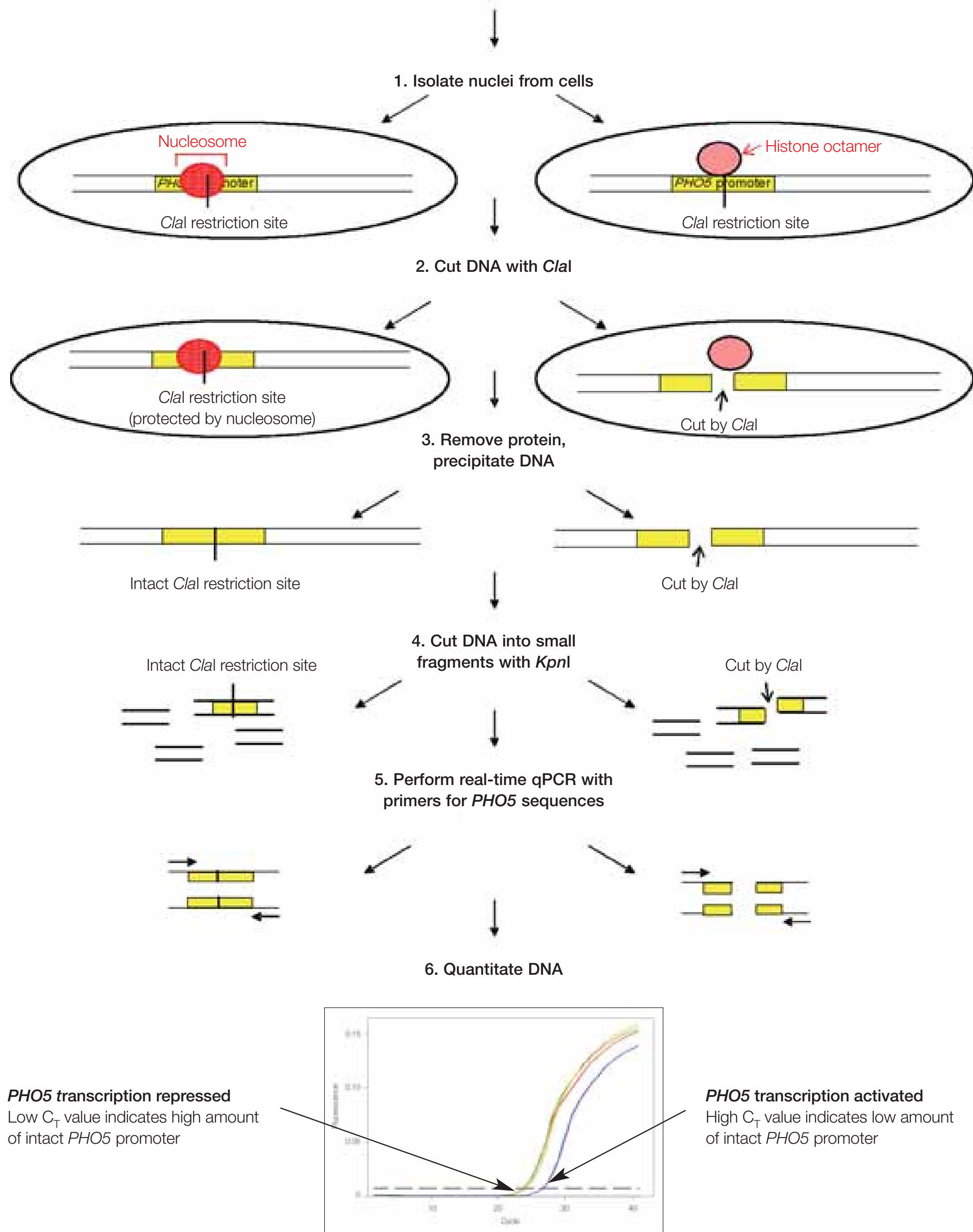

Fig. 1. Workflow for *Cla*I accessibility assay and quantitation by real-time qPCR.

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(5'-CAGTTGCGTTCTATGCGAAA-3') amplify a control region, a 150 bp fragment that lacks a *Cla*I site. The thermal cycling protocol used was:

1. Incubate 10 min at 95.0°C.
2. Incubate 15 sec at 95.0°C.
3. Incubate 1 min at 60.0°C.
4. Read plate.
5. Repeat steps 2–4 for 40 additional cycles.
6. Incubate 7 min at 72.0°C.
7. Perform melting curve function: From 65.0°C to 90.0°C, read every 0.2°C; hold for 1 sec between reads.
8. Incubate 7 min at 72.0°C.

Analysis was performed using Opticon Monitor™ software, version 1.04. The slope of the standard curve for each PCR product was used to calculate the qPCR reaction efficiency, $\xi = e^{(-1/\text{slope})}$. Samples repressed for *PHO5* expression, which contain an intact *PHO5* promoter, were used to generate the standard curves. Here, the assumption was made that products amplified by a given primer pair would have the same amplification efficiency for all reactions. This assumption would hold true for cells grown under either repressing or activating conditions. The fraction (F) of uncut DNA present in the digested samples was calculated using the equation:

$$F = \xi^{-\Delta C_T}$$

where ΔC_T is the difference between values for the threshold cycle (C_T) measured for the *Cla*I-digested and for the untreated samples (Boeger et al. 2003).

Results and Discussion

For cells grown under repressing conditions, real-time qPCR amplification of the undigested and *Cla*I-digested *PHO5* promoter region (containing a *Cla*I site) showed no difference in the amount of amplified product (Figure 2A). The mean threshold cycles (C_T) for undigested samples and digested samples were 23.98 ± 0.22 and 23.94 ± 0.20, respectively (Table 2), consistent with a model that depicts this region as protected by nucleosomes under repressing conditions. For cells grown under activating conditions, however, the *Cla*I-digested samples showed decreased amplification (Figure 2A), as shown by a higher mean C_T (26.50 ± 0.07) compared to the undigested samples (24.23 ± 0.14) (Table 2). These data suggest that the DNA has become exposed and accessible to *Cla*I. Samples amplified using primers for a control region lacking the *Cla*I site showed no difference in signal with or without *Cla*I digestion and under both activating and repressing conditions (Figure 2B and Table 2).

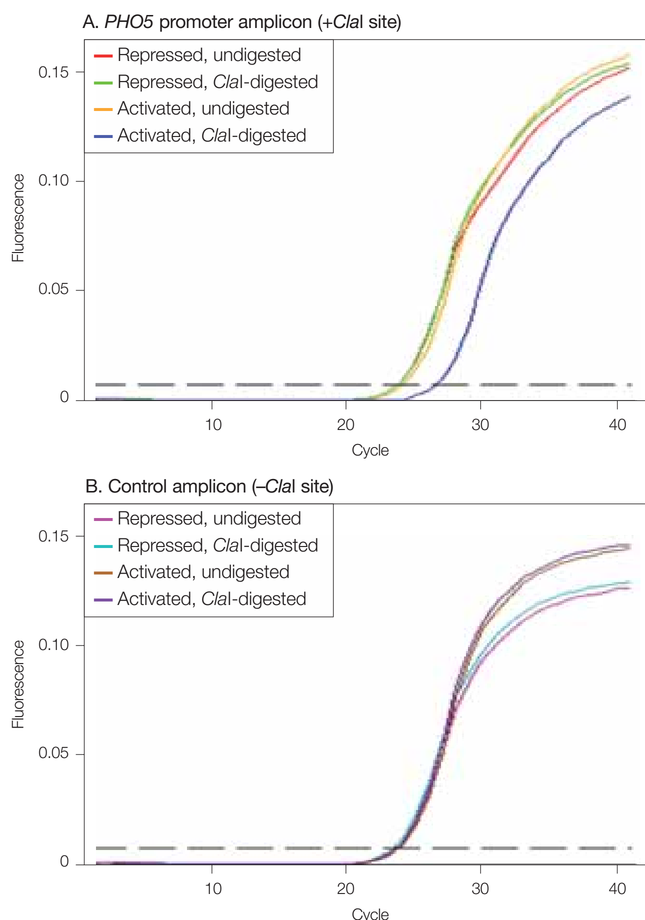

Fig. 2. Representative quantitation curves for the *Cla*I accessibility assay. A, amplification with primers for the *PHO5* promoter containing a *Cla*I restriction site from yeast grown under repressing conditions for *PHO5* expression or under activating conditions. B, amplification with primers for a control region lacking a *Cla*I site from yeast grown under repressing conditions for *PHO5* expression or under activating conditions.

Table 2. C_T values for amplification of the *PHO5* promoter region containing a *Cla*I site or a control region lacking a *Cla*I site. Samples were assayed in triplicate by qPCR after digestion with *Cla*I or no treatment with *Cla*I (undigested). Values shown are mean C_T ± SD (standard deviation).

	Amplicon	
	PHO5 Promoter (+*Cla*I Site)	Control (–*Cla*I Site)
Repressed		
Undigested	23.98 ± 0.22	23.47 ± 0.18
*Cla*I-digested	23.94 ± 0.20	23.29 ± 0.06
Activated		
Undigested	24.23 ± 0.14	23.70 ± 0.08
*Cla*I-digested	26.50 ± 0.07	23.50 ± 0.10

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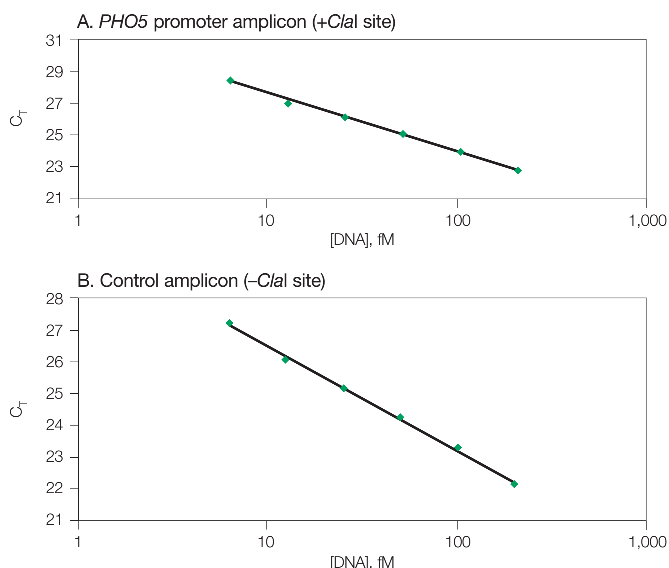

Fig. 3. Standard curves generated from C_T values for 2-fold dilution series. A, amplification of undigested sample with primers specific for the *PHO5* promoter containing a *Cla*I site; B, amplification of a control region lacking a *Cla*I site. R^2 values were 0.998 for both curves.

To determine relative differences in restriction endonuclease accessibility, we calculated qPCR efficiencies for both PCR products from standard curves generated from a 2-fold dilution series of undigested samples (Figure 3). For the primer pair amplifying the *PHO5* region with a *Cla*I site, the reaction efficiency was 1.84 (Table 3). For the primer pair amplifying the control region lacking the *Cla*I site, the reaction efficiency was 2.02 (Table 3). The reaction efficiencies were used to determine the fraction (F) of uncut DNA after *Cla*I digestion compared to total uncut DNA before *Cla*I digestion. As shown in Table 4, we observed a 75% increase in *Cla*I accessibility of the *PHO5* promoter with the *Cla*I site under activating conditions, and essentially no change in *Cla*I accessibility under repressing conditions.

Table 3. C_T values used to calculate amplification efficiency (ξ).

	Amplicon	
Amount of DNA (fmol)	***PHO5* Promoter (+*ClaI* Site)**	**Control (–*ClaI* Site)**
20	22.76	22.14
10	24.00	23.32
5	25.09	24.27
2.5	26.21	25.16
1.25	27.17	26.09
0.625	28.57	27.20
Efficiency (ξ)	1.84	2.02

Table 4. Relative accessibility of the *Cla*I site in the *PHO5* promoter. The fraction (F) of uncut *PHO5* promoter DNA after *Cla*I digestion was normalized to total uncut DNA in undigested samples (no *Cla*I digestion). Shown are mean values ± SD.

	Amplicon	
***PHO5* Expression**	***PHO5* Promoter (+*ClaI* Site)**	**Control (–*ClaI* Site)**
Repressed	1.03 ± 0.14	1.14 ± 0.15
Activated	0.25 ± 0.02	1.16 ± 0.07
% Change	~75%	—

Conclusions

qPCR using SYBR Green detection chemistry provides a robust and flexible solution for evaluating access of the *Cla*I restriction endonuclease to the chromosomal *PHO5* promoter DNA. We have shown that qPCR is a valuable tool for the analysis of chromatin transitions.

Compared to traditional Southern blot analysis, the qPCR approach has several advantages, including smaller sample requirements, higher throughput, improved specificity and sensitivity due to a wide dynamic range, and excellent reproducibility.

References

Almer A et al., Removal of positioned nucleosomes from the yeast *PHO5* promoter upon *PHO5* induction releases additional upstream activating DNA elements, EMBO J 5, 2689–2696 (1986)

Boeger H et al., Nucleosomes unfold completely at a transcriptionally active promoter, Mol Cell 11, 1587–1598 (2003)

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