Chromatin structure of the *Saccharomyces cerevisiae* DNA topoisomerase I promoter in different growth phases

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We have determined the chromatin organization of the *Saccharomyces cerevisiae* DNA topoisomerase I promoter. Three nucleosomal core particles have been mapped at nucleotide level over the promoter region, encompassing the presumptive TATA sequence and the two RNA initiation sites; the most upstream nucleosome particle forms on to a 29 bp-long poly(dA-dT) element. This simple organization remains constant throughout both the logarithmic and the linear phase of growth, with the exception of an increased accessibility to micrococcal nuclease of the nucleosome covering the TATA box and the RNA initiation sites during the diauxic shift (the switching from the fermentative to the respiratory metabolism) in parallel with an increase of the DNA topoisomerase I mRNA. In addition, a strong disorganization of the bulk chromatin structure in the late stationary phase is also reported.

INTRODUCTION

The nucleosomal particle has long been considered a barrier to the process of transcription, its function being supposedly limited to preventing the access of regulatory proteins and of the transcription machinery to promoter sequences. This view of nucleosomes as a structural barrier is insufficient to explain the different behaviours and organization schemes emerging for promoters from a variety of sources. Models of nucleosomes as regulatory elements have been proposed [1–5], based essentially on the idea that when DNA is laid on the surface of a histone octamer [nucleosomal core particle (ncp)] it is spatially organized in a toroidal superhelix, conferring new properties for the access of and interaction with regulatory proteins and RNA polymerase.

Ncps may be released or modified upon induction, as exemplified by the PHO5 (acid phosphatase) promoter [6], allowing access of transcription factors. A paradigmatic example is provided by the Pho4 protein, whose access to a critical target site in the promoter is supposedly prevented by a nucleosome [7]. In other instances, induction causes (or is mediated by) changes in nucleosome conformation or position, as shown for hormone interaction [8], for factor-mediated chromatin remodelling of specific promoters [9], and for enhancement of the stimulation of transcription by the oestrogen/oestrogen receptor by the presence of a nucleosome in a defined position upstream from the transcription start [4]. Consistent with such a positive role of nucleosomes is the observation that the H4-terminal tails are required for the activation of promoters in yeast [10]. These recent findings enrich the array of the possible functions of nucleosomes in the regulation of transcription. However, in the majority of the instances reported so far, the presence of nucleosomes at promoters has been associated with repressive effects on their activity [11–16]. All data and models mentioned above refer to inducible promoters, with the exception of the work related to the RNA polymerase III-transcribed 5S gene [15]. A detailed analysis of the chromatin organization of a typically constitutive, RNA polymerase II-served promoter is lacking. Knowing the position, and possibly the function, of the nucleosomes of a constitutive promoter is therefore of interest to help answer the question of whether nucleosomes are negative barriers or structural enhancers/regulators.

DNA topoisomerase I (TOP1) serves a basic house-keeping cellular function and its expression is thought to be constitutive [17,18], at least in yeast. Both the protein and the corresponding mRNA are indeed present throughout the life of the cell and no observation of regulation of its expression has been reported. Preliminary low-resolution mapping [19] of the chromatin region encompassing the promoter and the coding part of the TOP1 gene suggested the presence of a few regularly arranged localized ncps. The nucleotide-level localization of these ncps is reported here, showing multiple, alternative positions.

As mentioned above, TOP1 expression is generally considered not to be regulated. We report that the levels of its mRNA are modulated in different stages of growth, increasing in correspondence with the metabolic change that takes place at the diauxic shift (the switch from fermentation to respiration [20,21]). Changes of nucleosomal organization accompany these variations in mRNA levels. At later stages of growth there is a marked decrease in TOP1 mRNA levels, when the bulk chromatin appears strongly disorganized.

MATERIALS AND METHODS

Nystatin was purchased from Sigma, zymolyase from Seikagaku, Proteinase K and micrococcal nuclease from Boehringer Mannheim and Taq polymerase from Promega. The *Saccharomyces cerevisiae* W303-1a strain (*Mat*a, *ade*2-1, *ura*3-1, *his*3-11, 15 *trp*1-1, *leu*2-3,112 *can*1-100), also named RS188, was a kind

Abbreviations used: hsp, heat-shock protein; MMTV, murine mammary tumour virus; MN, micrococcal nuclease; ncp, nucleosomal core particle; TOP1, topoisomerase 1.

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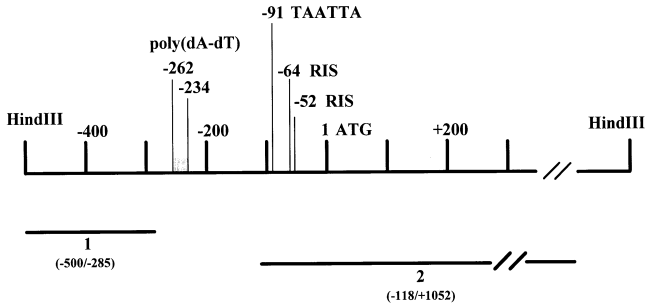

Figure 1 Map of the *S. cerevisiae* DNA TOP1 gene

Localization of the indicated functional sites [RIS, presumptive TATA box, ATG and poly(dA:dT)] is reported as described [17]. The borders of the TOP1 gene are defined by the two indicated *Hin*dIII sites, 3787 bp apart. The sequences are numbered relative to the ATG (A = position +1). The indicated probes were used in the indirect end-labelling mapping (Figure 5, probe 1) and in the Northern analysis (Figure 4, probe 2).

gift from Dr. Rolf Sternglanz (Stony Brook, NY, U.S.A.). Cells were grown in minimal medium supplied with the specific amino acids required by this strain.

Spheroplast permeabilization by nystatin

Yeast spheroplasts from exponentially growing cells (A_{600} 0.3–0.5/ml) or from different stages of growth were prepared with zymolyase, as described [22]. Spheroplasts were centrifuged and resuspended in nystatin buffer containing 50 mM NaCl, 1.5 mM $CaCl_2$, 20 mM Tris/HCl pH 8.0, 1 M sorbitol and 50 μg/ml nystatin.

Chromatin analysis by micrococcal nuclease (MN) *in vivo*

Low-resolution nucleosome mapping was performed by MN cleavage followed by indirect end-labelling [23] using probe 1 (Figure 1, position −500/−285). A shorter probe (70 bp long, −500/−430) was also used to confirm this nucleosome mapping, with identical results (not shown). Nystatin-treated spheroplasts were reacted with increasing amounts of MN. After 10 min at 37 °C, the MN reaction was stopped by 1 % (w/v) SDS, 5 mM EDTA and 100 μg/ml of Proteinase K. Purification, electrophoretic and Southern-blot analysis were carried out as in [19].

High resolution mapping *in vivo* was performed according to [24,25], as follows: nystatin-treated spheroplasts were incubated with MN at the concentrations indicated in Figures 2 and 3. The analytical process is detailed in [24,25]. Schematically, the DNA of MN-treated chromatin was purified, primer-extended under PCR conditions, using the oligonucleotides indicated below, and analysed by sequencing gel electrophoresis. The oligonucleotides used were: oligo #1 (for mapping of nucleosome +1, Figure 2): 5′CGACGACGCATTTTGATATA3′, from position −142 to −116; oligo #2 (for nucleosome −2, Figure 3): 5′GGTTCAA-CACTAACACGAGCGCAATA3′, from position −441 to −422.

RNA preparation and Northern analysis

RNA was prepared following the rapid method of Schmitt et al. [26]. Samples (5 ml) were collected at different moments of the culture growth and centrifuged. The RNA was phenol-extracted at 65 °C. Northern blot and hybridization were performed by standard procedures [27] using probe 2 (Figure 1, position −118/+1052).

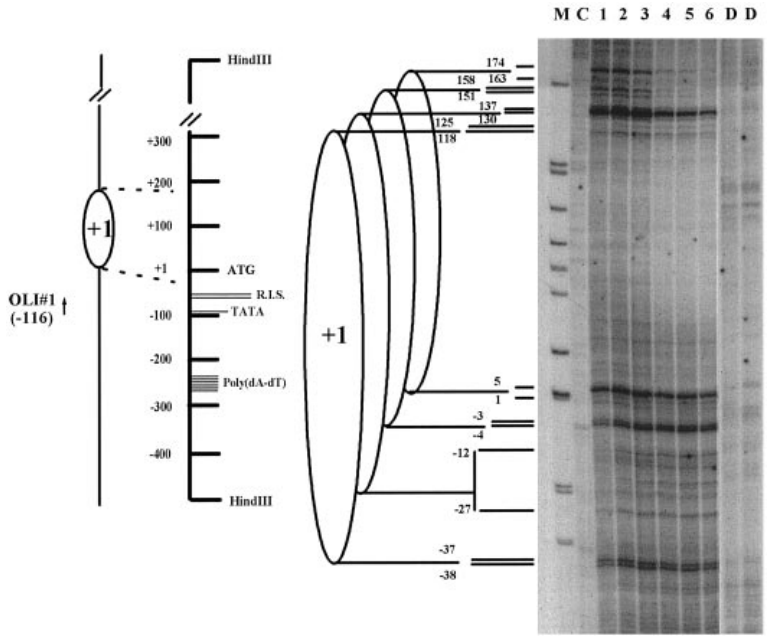

Figure 2 Localization of nucleosome positions by primer extension on purified oligonucleosomal DNA: nucleosome +1

Nucleosome +1 was localized at nucleotide level by elongation of oligo #1 by PCR, as detailed in the Materials and methods section. Controls: M, marker pBR322/MspI; C, *S. cerevisiae* chromosomal DNA, untreated, elongated with oligo #1 (arrow on the left side of the scheme, starting at position −116; the scarcity of background due to elongation pausing is noticeable); D, chromosomal DNA digested *in vitro* with 0.5 and 1 unit of MN. Lanes 1–6, chromatin digested *in vivo* with 2.5, 5, 10, 20, 40 and 60 units of MN. The borders relevant for mapping nucleosome +1 are indicated on the left side of the gel and are numbered relative to the ATG.

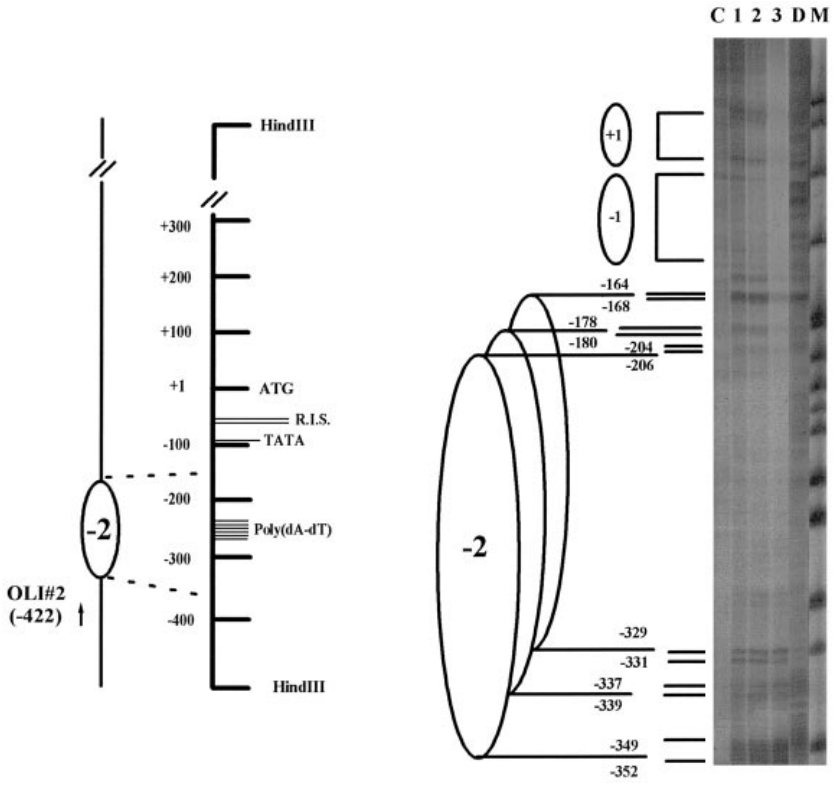

Figure 3 Nucleosome −2

Analytical procedures and display as for Figure 2. Elongation from oligo #2, starting at position −422.

RESULTS

Chromatin organization *in vivo* of the TOP 1 promoter at high resolution: alternative positions of the ncps in the promoter area

A schematic map of the functional organization of the TOP1 promoter [17] is shown in Figure 1. The low-resolution mapping reported in [19] and in Figure 5 (this work) was used as a basis to programme the nucleotide-level localization of ncps. This analysis consists in the identification of the ncp borders as revealed by limited MN treatment *in vivo*. The digestion with increasing amounts of MN nuclease was performed on yeast cells using nystatin permeabilization, a procedure which allows penetration of enzymes into spheroplasts (see the Materials and methods section and [19]). The treatment was followed by primer extension of the purified chromosomal DNA starting from an oligonucleotide external to the nucleosome. For nucleosome +1 (Figure 2) the results show: (i) a completely protected central area spanning about 115 bp, between map positions 5 and 118; and (ii) multiple borders encompassing four helical turns on both sides that can be divided into four groups as indicated. Most of these borders are quite sharp, others (see for instance the lower borders of the second particle from the bottom) are more disperse, probably due to partial sequence specificity of the enzyme. These borders are coupled to define a bona fide ncp by matching their distances to 150 ± 5 bp. Numerical matching is not performed to the standard 146 bp value, considering that for the very nature of this assay the MN digestion is not pushed to its limit. Minor imprecisions may occasionally arise (as for the second particle) due to dispersion of the MN cleavages (for the reason mentioned above). The position(s) of ncp family +1, as it results from these analyses, is the following: multiple occupancy on four different, alternative positions occupying a central common area of 115 bp characterized by a clear footprint, plus alternative occupancies spanning four lateral helical turns on either side, as depicted in Figure 2.

Figure 3 describes the same analysis for the ncp family −2. In this case at least three (and possibly one more) alternative nucleosomal particles are observed. The overlapping of the upper borders of ncp family −2 with the lower borders of the ncp family −1, whose footprinting is indicated in the upper part of the gel (square bracket), makes the attribution of additional particles uncertain.

TOP1 RNA levels as a function of the growth phase

TOP1 expression is considered to be constitutive. Its mRNA is indeed present throughout the cell life. However, we observe that the TOP1 mRNA levels change during the growth of the cell culture. RNA was prepared from cells taken at different stages of growth (Figure 4) and the amount was determined with an LKB spectrophotometer. Growth-phase-related variations were observed. In particular, TOP1 mRNA increases by a factor of two at a cell density of A_{600} 1.6–1.8/ml relative to the initial stage of growth. In a typical growth curve under the conditions used (results not shown), at the A_{600} values of 1.6–1.8, a decrease in the growth rate occurs due to nutrient limitation corresponding to glucose exhaustion. It is known that at this stage cells undergo diauxic shift and switch from fermentation to a respiratory pathway [20,21]. After the shift the TOP1 mRNA continues accumulating up to a 5-fold increase when approaching the

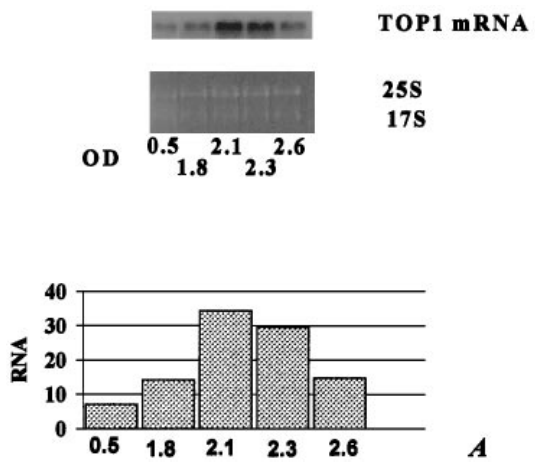

Figure 4 TOP1 RNA levels during growth

(**A**) Total RNA, extracted from cells grown in minimal medium and collected at the indicated growth stages (A_{600} 0.5, 1.8, 2.1, 2.3, 2.6/ml) were analysed by Northern hybridization using probe 2. The amount loaded is 10 μg/lane. The two major rRNAs are indicated for quantitative reference. The upper panel shows specific hybridization with the probe containing the TOP1 coding sequence. (**B**) Densitogram of TOP1 RNA.

stationary phase, while at the very late stages of growth (A_{600} 2.6/ml, two days after inoculation) it decreases sharply, in parallel with the general reduction that characterizes the transcription rate at this stage [21].

Chromatin structure of the TOP1 promoter as a function of the growth phase

In order to investigate whether the modulation of mRNA levels correlates with changes in chromatin, samples of cell were taken for chromatin analysis in parallel with the cells used for RNA extraction. Figure 5(A) shows a comparison between chromatins prepared *in vivo* at A_{600} 0.5/ml (exponential growth phase) and at A_{600} 1.8/ml (diauxic shift), following the procedure described. The low-resolution chromatin analysis was performed by indirect end-labelling relative to the *Hin*dIII site located upstream of the promoter. Hybridization with probe 1 (215 bp long), located immediately adjacent to the restriction site, defines the areas protected from nuclease cleavage and the areas accessible to MN. The chromatin organization was confirmed by an analysis performed on the same region from the opposite orientation. The probe used was an internal fragment from position +479 to +637 (results not shown). Increased accessibility to MN is detected at A_{600} 1.8/ml, specifically located on the ncp family −1 (Figure 5, filled triangle) which encompasses the TATA box and the RNA initiation sites (RISs), suggesting that in parallel with the RNA increase this ncp family is partially destabilized.

It is known [21] that carbon starvation induces diauxic transition. To correlate the specific alteration in the ncp −1 with passage through the diauxic shift, we analysed the chromatin organization of a culture after glucose depletion. Cells grown on medium containing 3% glucose were washed twice with water, resuspended in the same volume of fresh YPD [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose] medium containing 0.05% glucose, and samples for chromatin analysis were

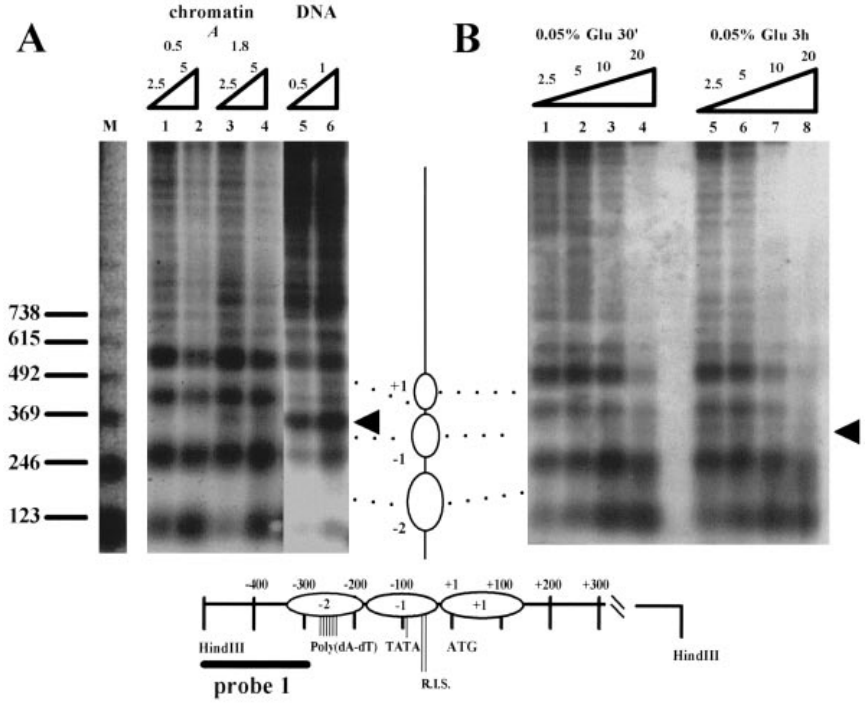

Figure 5 Localized chromatin remodelling induced by diauxic shift at low resolution

(**A**) MN cleavage sites on chromatin *in vivo*, mapped relative to the 215 bp-long probe 1 located upstream of the promoter (see Figure 1). Digests of chromatin *in vivo* are shown in lanes 1–4; 0.5 and 1.8 are values of A_{600}/ml. Control: digest of purified genomic DNA produced by the amounts of MN (0.5 and 1 units) indicated on top of lanes 5 and 6. The increased accessibility to MN of ncp −1 is indicated by the filled triangle. M, marker (123 bp multiples). The scheme at the bottom allows localization of the presumptive nucleosomes. (**B**) MN cleavage sites on cells grown in 0.05% glucose for 30 min (lanes 1–4) and for 3 h (lanes 5–8). The amounts of MN are indicated above the triangles.

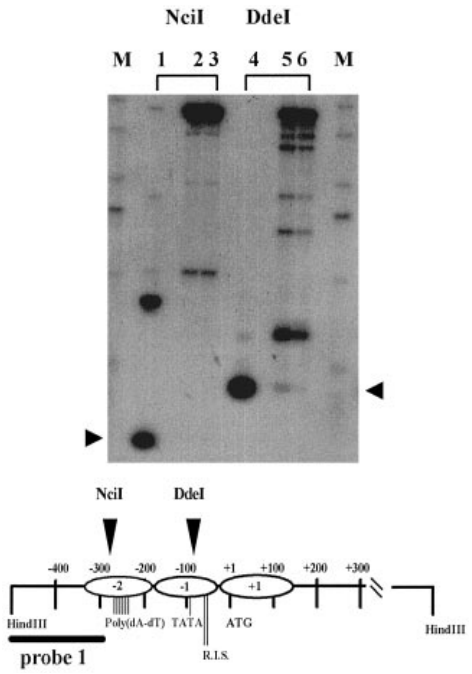

Figure 6 Restriction enzymes accessibility induced by diauxic shift

Spheroplasts from cells grown in 0.05% glucose for 3 h (lanes 2 and 5) or in 3% glucose overnight (lanes 3 and 6) were treated with 20 units of the indicated restriction enzymes. Lanes 1 and 4: samples digested *in vitro*. See text for explanation of arrowheads. Secondary restriction: *Hin*dIII. M, 1 kb ladder. The linear map below shows the positions of the *Nci*I and *Dde*I restriction sites (−285 and −84 respectively). The fragments relative to these sites are indicated by arrowheads.

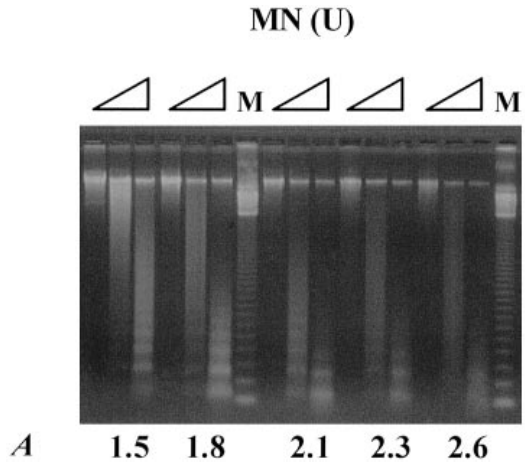

Figure 7 Nucleosomal spacing of bulk chromatin as a function of the growth phase

Spheroplasts were obtained at various growth phases (as indicated at the bottom of each group of three lanes where the A_{600} value is given) and treated respectively with the following increasing amounts of MN: 0, 3, and 10 units. M, marker (123 bp multiples).

taken at 30 min and 3 h (Figure 5B). Under these conditions the increased accessibility to MN over the ncp −1 (Figure 5, arrowhead) is barely visible in the 30 min sample and is clearly evident after 3 h, thus confirming that upon diauxic shift the ncp −1 changes its structure or its frequency of occupancy. During the initial stage of this analysis, we have actually interpreted the −1 region as a nucleosome-free area. The increased accessibility was also confirmed by using the restriction enzyme *Dde*I on cells grown in 0.05% or 3% glucose (Figure 6, lanes 5 and 6 respectively). The cleavage in lane 5 (arrowhead), absent under 3% glucose conditions, indicates a partial destabilization of nucleosome −1, in agreement with the MN result in Figure 5(B), lanes 5–8 (arrowhead). Nucleosome −2 does not change its conformation and prevents cleavage by *Nci*I (lanes 2 and 3) under all conditions tested. This result highlights the fact that controlled growth conditions are important variables to be taken into account when interpreting yeast chromatin analyses.

These results indicate that the onset of stationary phase causes alterations of both chromatin organization and transcription. This is, in principle, to be expected, given that the stationary phase constitutes a major alteration of overall metabolic functions. Previous reports [28] describe changes in the overall chromatin conformation in late stationary-phase cells. Figure 7 shows the nucleosomal spacing of bulk chromatin as a function of the growth phase. The standard spacing is observed in logarithmically growing cells up to A_{600} 1.5, before the levelling off (at A_{600} 2.1) of the culture growth. From this stage on, chromatin becomes progressively more accessible to MN, and the nucleosomal pattern is lost. This general disorganization also specifically characterizes the TOP1 promoter (Figure 8). Whether this is due to a general loss of nucleosomes, to a randomization of positions or to chemical and/or conformational changes of the histone octamer remains to be established.

DISCUSSION

No single rule applies to the relationship between nucleosomes and regulatory proteins in promoters. In the yeast PHO5 promoter, for instance, binding of the regulatory protein Pho4 to the upstream activating sequence (UAS) 2 is prevented in the repressed state by a nucleosome, while binding is allowed to upstream activating sequence (UAS)1, a site which resides in a constitutively nucleosome-free region. In this system, the presence of nucleosomes correlates with the repressed state, and activation of transcription requires nucleosome 'dissolution' [29]. In other systems, activation is mediated by a modification of the structure of the regulatory nucleosome [8,30], caused by the inducer [8], or is accompanied by a protein-mediated chromatin remodelling, as exemplified in the heat-shock protein (hsp) 26-GAGA factor system [9]. For a recent review see [31]. The role of nucleosomes in this complex interplay remains an important question. For the organization of chromatin at regulated promoters at least four different classes of models can be outlined. A first model which requires the removal of one or more nucleosomes (model PHO5, [6]). A second model implies remodelling of nucleosomal structure without nucleosome removal {murine mammary tumour virus (MMTV) model [8]}. A third alternative mechanism is provided by the hsp26 promoter, where the alternative positions of strategically located nucleosomes change upon the inducing event (HSP26 model [9]). A fourth organization scheme is provided by the *Hansenula polymorpha* MOX promoter, in which nucleosomes tightly and completely cover the promoter region occupying every alternative, rotationally phased position (MOX model [32]). In this promoter, at least one regulatory Adr1-like protein binds to nucleosomal DNA. Complete occupancy of the regulatory region by nucleosomes is obtained in this system, which exploits the strength of the rotational information in a way similar to that described for the continuous occupation by nucleosomes of the yeast 5S gene [33].

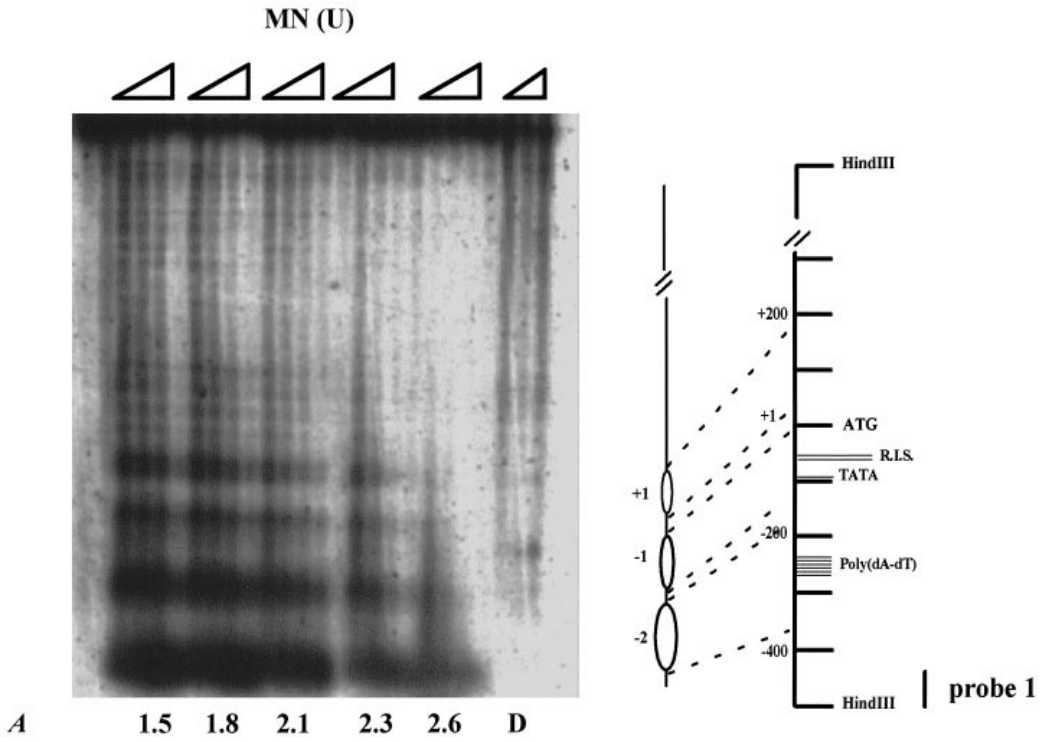

Figure 8 Variation of the cromatin structure of the TOP1 promoter as a function of growth phase

An aliquot of the same chromatin preparation as that analysed in Figure 7 was digested with *Hin*dIII and indirect-end-labelled with probe 1 (see Figure 1). MN amounts: 1, 3, 10 and 15 units (U) for each group of four lanes. A_{600}, gel analysis and display of the data are as in Figure 7. Attribution of the protected areas is as in Figure 5.

Multiple alternative translational positions

Much less is known about constitutive promoters. The promoter analysed here provides a simple organization scheme: a regular nucleosome array over the regulatory elements. The three ncps which localize on the TOP1 promoter occupy multiple positions (Figures 2 and 3). Such multiplicity is characterized by the alternative occupation of different translational positions along a unique rotational frame. A similar behaviour has been observed in several yeast gene systems {*S. cerevisiae* alcohol dehydrogenase (ADH) 2 [30], 5S [33], autonomously replicating sequence (ARS) 1 [25], *H. polymorpha* [32]} for a total of 11 nucleosomes mapped at nucleotide level with several different methods. A summary of the various techniques used with coherent results in these analyses can be found in the literature [24,33]. The number of alternative positions covers a wide spectrum of possibilities, going from one major position for ncp -3 in the MOX promoter to at least 14 different positions on the 5S gene. The observed average is 4–5 positions; the three ncps which organize the structure of the TOP1 promoter are not dissimilar.

Multiplicity is energetically plausible, due to the fact that in ncps the DNA–protein contacts take place on repetitive helically phased signals. Therefore ncps may locate on DNA on alternative, partially overlapping positions. This type of organization suggests that in yeast promoters it is not relevant what sequence engages on what part of the histone octamer, as long as a given DNA tract is engaged with an octamer and as long as the rotational phase (i.e. the correct exposure of the DNA) is kept coherent. This view is in agreement with the results of a study on the effect of nucleosome phasing sequences and DNA topology on nucleosome spacing [34]. The present analysis concludes that DNA sequences, as such, are unlikely to be decisive for translational positions *in vivo*, being, on the contrary, likely determinants of 'fine tuning' through rotational setting.

Chromatin structure and TOP1 expression in different growth phases

S. cerevisiae TOP1 is considered to be a typical constitutive gene [17], as no specific inducers or repressors of its transcription are known. We observe that, in addition to a continuous constitutive presence, its mRNA undergoes quantitative variations. A peak is reached at the late stages of growth before the onset of the stationary phase induced by both general nutrient limitation and glucose depletion. The relevance of this increase could fit with the reported involvement of TOP1 in the generalized repression of most genes following the diauxic shift [20]. In correspondence with this increased transcriptional activity, local chromatin remodelling is observed, which is detected by an increased accessibility of the strategically located ncp -1. Changes in the chromatin structure at the glyceraldehyde-phosphate-dehydrogenase (TDH) 3 promoter, analysed by DNaseI, were previously detected as a function of the growth stage [35]. In line with this evidence, and that reported above for the second and third models, we interpret this finding as an indication of nucleosome remodelling as a function of increased transcription. Whether this conformational variation is a cause or an effect cannot be defined at this stage of investigation. In growth stages corresponding to a lower transcription rate of the TOP1 promoter, this modification is not detected, presumably because of its low frequency of occurrence. This would imply a negative

role for ncp −1, as only a subset of configurations that lack it would be engaged in transcription. The variable accessibility to MN of the sequence interacting with ncp −1 as a function of the growth stage and of specific metabolic conditions, highlights the dynamic nature of chromatin organization. In conclusion, a bona fide constitutive TOP1 promoter does not appear to behave differently from typically regulated ones, such as MMTV or GAGA responsive promoters.

A further remodelling event is observed in late stationary phase (Figure 7). An increased accessibility of MN to nuclear DNA, together with a loss of nucleosomal pattern, suggests a general disorganization of the chromatin. This disorganization parallels the loss of nucleosomal regularity over the TOP 1 promoter. Further studies would be necessary to determine whether other genes behave in the same way at this stage of growth.

Role of poly(dA-dT)

A 29 bp-long poly(dA-dT) stretch is present in the TOP1 promoter between the upstream positions −234 and −262. The effect of this structurally rigid [36] homogeneous stretch is likely to be important in yeast gene expression, given the frequency of its occurrence in upstream sequences [37,38]. Whether this presumptive regulatory role is played through interaction with a specific poly(dA-dT)-binding protein [39] or by affecting chromatin structure is still a controversial point.

Clear evidence has been obtained that the presence of the poly(dA-dT) sequences stimulate general control non-repressed (GCN) 4-activated transcription, that the activity of poly(dA-dT) increases continuously with its length and that these sequences improve accessibility of the Gcn4 binding site *in vivo* [40]. The marked stimulatory effect caused by the increase in the length of the poly(dA-dT) stretch on alcohol dehydrogenase (ADH) 2 expression in naturally occurring *S. cerevisiae* mutants has long been known [41,42]. Thus, poly(dA-dT) does have an effect on transcription. Much less clear is the effect of poly(dA-dT) stretches on nucleosome localization. *In vitro* reconstitution data initially indicated that dA-dT tracts prevented the formation of nucleosomes [43,44], but experiments performed under milder reconstitution conditions showed that poly(dA-dT) and other synthetic polydeoxynucleotides containing oligoadenosine attracts nucleosomes easily [45,46]. Whatever the conclusion of these controversial *in vitro* findings, we observe that on the TOP1 promoter a well-defined nucleosome −2 forms *in vivo* on to a poly(dA-dT) stretch. Considering the possibility that regulatory proteins may bind on DNA sequences which are already engaged in nucleosomal particles (as in the MMTV and MOX examples mentioned above), the regulatory role of poly(dA-dT) sequences may be explained without invoking nucleosome-exclusion effects.

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