Changing nucleosome positions *in vivo* through modification of the DNA rotational information

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The effects of the rotational information of DNA in determining the *in vivo* localization of nucleosomal core particles (ncps) have been studied in the *Saccharomyces cerevisiae* 5 S rRNA repeat gene. The distribution of the phased series of flexibility signals present in this DNA has been altered by inserting in its centre a 25 bp tract. The effects of such alteration on the *in vivo* distribution of the helically phased, alternatively located ncps have been determined relative to a reference 21 bp insertion mutant. The results show that the answers provided *in vitro* and *in vivo* by the yeast 5 S rRNA gene sequence to specific modifications of the DNA rotational frame are similar, thus pointing to the relevance of DNA rotational information *in vivo*.

INTRODUCTION

The position of nucleosomes plays an important role in the regulation of transcription ([1–4], reviewed in [5]) and replication (reviewed in [6,7]). Nucleosomal core particles (ncps) form on essentially every eukaryotic DNA sequence with a wide range of affinities. Based on *in vitro* reconstitution free energies relative to a synthetic reference DNA fragment [8], a hierarchy of sequences has been established into which every biological sequence can be accommodated. The answer to the question of how ncps select defined locations on a potentially infinite set of sequence combinations has remained elusive for a long time. A satisfactory answer implies that the preferential interaction is determined by the DNA conformational properties provided by a consensus distribution of helically phased sequence combinations [9,10]. Helically coherent conformational properties define the rotational information of DNA.

If the positioning of a large protein complex is determined by repetitive quasi-isoenergetic interactions with DNA, it is likely to undergo informational ambiguity, possibly leading to multiple alternatives. Thus, nucleosomes may be expected to distribute in families formed by a small number of alternatively located individuals shifted by one helical turn.

It has been pointed out that a strong rotational setting may not be sufficient to position nucleosomes *in vivo* [11]. Unique, unambiguous positioning may be obtained by several mechanisms (boundary effects exerted by proteins or by specific DNA sequences, kinetic mechanisms, specific DNA sequences providing unambiguous translational information for the interaction, etc. (reviewed in [12,13]), and by covalent modifications of DNA (i.e. CpG methylation, shown to prevent histone octamers from interacting with an otherwise high-affinity positioning sequence in the promoter region of the chicken adult β-globin gene [14]). Single [6,7,15] or low-dispersion [16,17] positions have been described. The unique location obtained on a palindromic sequence derived from human α-satellite has recently been exploited to determine the crystal structure of an ncp at 2.8 Å resolution [18].

On the other hand, multiple positioning has been observed in several instances in both animal [19,20] and yeast systems [21–24]. The gene system on which the highest number of multiple ncps has been observed, both *in vivo* and *in vitro*, is the *Saccharomyces cerevisiae* 5 S rRNA [25], in which at least 16 ncps have been described, essentially covering the whole sequence by alternatively located particles. This set of alternative particles offered the possibility of establishing the function of the DNA rotational information in localizing ncps and has set an experimental system for testing the consequences of the variation of rotational signals *in vivo*. The reconstitution and localization analyses were carried out *in vitro* on 280, 305 and 312 bp 5 S gene-containing fragments; the *in vivo* analyses were performed on the chromosomal copies of this gene. Comparison of the *in vitro* and *in vivo* occupancies has shown that ncps occupy *in vivo* the same multiple helically phased sites occupied *in vitro*.

The role of rotational information was tested by modifying this information and analysing the reconstituted ncp complexes. Modification was obtained by inserting in the centre of a 305 bp 5 S DNA one of two segments [26]: the first is 21 bp long (denoted *in21*), and the other is 25 bp (*in25*). These segments shift the repetition of the DNA bendability signals by a whole number of helical turns (namely, two turns) for *in21* or by an uneven number (namely two + about one-half turns) for *in25*. For *in21* the signals present on the sequence on one side of the insert remain in helical phase with the signals on the other side; for *in25*, these signals go in counter-phase relative to each other.

The results of this analysis are independent of the nature of the inserted sequence (not shown) and have shown that, at least for the 5 S rRNA gene, the DNA rotational information is a major determinant for ncps positioning. In addition, the number of rotationally phased signals required for a stable particle was defined. The analysis of the helical pattern generated by hydroxyl radical degradation of the reconstituted ncps has shown that

Abbreviations used: ncp, nucleosomal core particle; MN, micrococcal nuclease; PE, primer extension.

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when a foreign sequence is inserted in the track of regular helically phased interactions, the ncps that contact the insert locally distort the regular helical pattern in order to keep binding on the flexibility track. The distortion is kept at a minimum and involves only three helical turns. The major result of this *in vitro* analysis is the following: the ncps that localize the helically uneven insert in their centre are unstable and do not form. Thus, short well-defined sequence insertions can be used to understand and to interfere with structural and regulatory phenomena involving nucleosomes. To do so, the first step necessarily consists of the determination of the effects of the alteration of the helical phase on ncps *in vivo*.

Here we present data on the consequences of the rotational alterations induced by the *in21* and the *in25* constructs when inserted in plasmids and analysed *in vivo*. The results show that the multiplicity of positions *in vivo* is altered by the insertion of the half-helical phase. The alteration is limited and local.

MATERIALS AND METHODS

Materials

Nystatin was purchased from Sigma, zymolyase was from Seikagaku, proteinase K and micrococcal nuclease (MN) were from Boehringer Mannheim, and Taq polymerase was from Promega.

Localization of ncps

This was performed as described previously [25–27]. *In vivo* analysis was performed according to the procedure described in detail in [27]. This procedure essentially consists of the following. (1) Spheroplast permeabilization by nystatin. Yeast spheroplasts from exponentially growing cells (A_{600} 0.3–0.5/ml) were prepared with zymolyase, 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. (2) Chromatin analysis by MN *in vivo*. High-resolution mapping *in vivo* was performed as follows: nystatin-treated spheroplasts were incubated with MN at the concentrations indicated in Figure 2. The analytical process is detailed in [25–27]. Schematically, the DNA of MN-treated chromatin was purified, primer extended using the oligonucleotides indicated below, and analysed by sequencing gel electrophoresis.

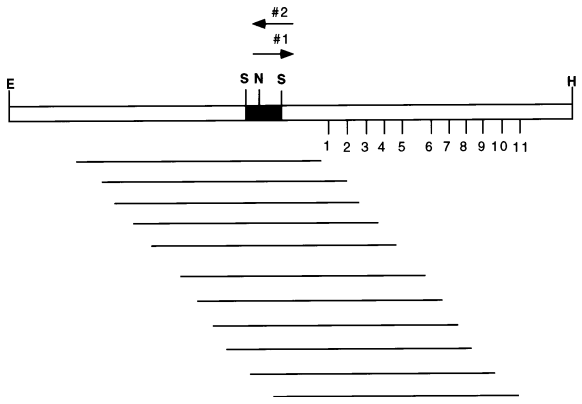

Figure 1 Localization of the ncps reconstituted on the *in21* mutant of the 5 S rRNA gene

This scheme describes the ncps localization on a construct made of a 305 bp *Eco*RI–*Hin*dIII DNA tract containing a single copy of the yeast 5 S rRNA gene in the middle of which a 21 bp DNA segment was inserted (see the Materials and methods section). The primers used for the relevant primer extensions (arrows; see the Materials and methods section) are indicated. The numbers refer to the borders of the ncp identified as described in the legend to Figure 2. Details of the mapping procedures and ncp sizes are in [25–27]. Abbreviations: E, *Eco*RI; H, *Hin*dIII; S, *Sau*3AI; N, *Nci*I and *Nco*I.

The *in21* and *in25* constructs consist of the 305 bp *Eco*RI–*Hin*dIII DNA tract of the pBB111F plasmid [29] containing a single copy of the yeast 5 S rRNA gene into which the sequence GATCCCCCATGGGTGGTGGGGTATA was inserted at the *Sau*3AI site (*in25*). The *in21* construct was obtained from *in25*, as described previously [26]. The primers # 1 and # 2 used for primer extension (PE) (respectively: 5′-GGTGGTGG-GGTATAGATCAA-3′ and 5′-TTGATCTATACCCCACCA-CC-3′) correspond to part of the insert sequence, and their locations are approximately indicated in the map in Figure 1 by arrows.

The experimental details of the *in vitro* ncp localization by MN digestion/PE are as follows. A total of 500 ng of each of the *in21* and *in25* *Eco*RI–*Hin*dIII fragments 5′-labelled at low specific activity was reconstituted at a rate donor ncp/acceptor DNA ratio of 8:1. After reconstitution, the fragments were digested (12 min, 37 °C) with MN (2 units/ml), and monomeric DNA was isolated on an agarose gel as described [26]. After a second purification on denaturing polyacrylamide, monomeric DNA was extended using primer # 1 (Figure 1).

The *in vivo* mapping based on the differential accessibility to restriction enzymes is described in the Results and in the legends to Figures 4 and 5.

RESULTS AND DISCUSSION

Mapping of *in vivo* nucleosomal positions by PE of monomeric DNA

The positions occupied by ncps upon *in vitro* reconstitution on *in21* and *in25* constructs have been previously determined by ExoIII analysis, by hydroxyl radical footprinting and by restriction cleavage of MN-digested DNA [26]. These low-background techniques cannot be used *in vivo*. Therefore, in order to determine the positions occupied *in vivo* and the frequency of their occupancy, we have performed MN digestion of permeabilized spheroplasts followed by PE of DNA isolated from purified monomer-sized ncps (see below) [25,27,28].

The validity of the MN accessibility/protection assay in nucleosome localization *in vitro* and *in vivo* is confirmed by the consistency of the data it provides with the data obtained with other analytical techniques (namely DNase I protection analysis, determination of ncp borders by ExoIII, hydroxyl radicals footprinting, and protection from restriction-enzyme attack [23,25–27]).

In21 and *in25* constructs were inserted into a vector containing an *S. cerevisiae* ARS1 origin of replication and were used to transform yeast cells. PE analysis of monomeric DNA generated *in vivo* by MN digestion is shown in Figure 2. The same technique has been used to map the positions occupied by ncps reconstituted *in vitro* on the *in21* and *in25* constructs. A direct comparison of the *in vivo*/*in vitro* positions is shown.

Two controls were performed. (1) The *in21* naked *Eco*RI–*Hin*dIII DNA fragment (10 μg) was mildly digested with MN (0.1 unit/ml), and a population of fragments about 146 bp long each was purified in parallel to monomeric DNA on both agarose and denaturing polyacrylamide gels. This population of fragments was extended with primer # 1 (Figure 2, lane N) in order to locate the sequence positions biased by the sequence specificity of MN. (2) A control for the elongation pausing was performed by extending naked DNA (the *in21* *Eco*RI–*Hin*dIII

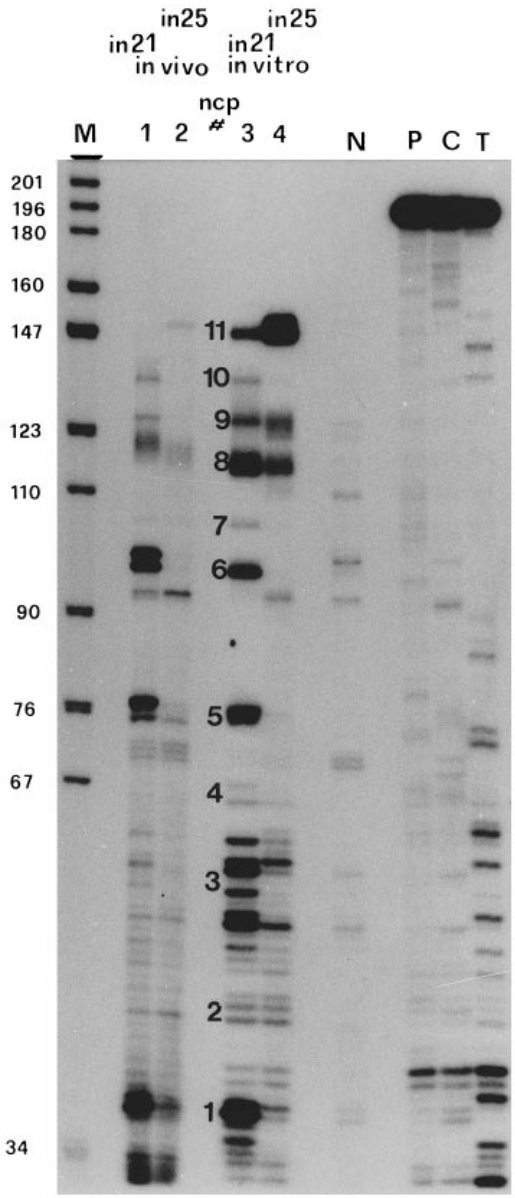

Figure 2 Localization of the borders of the ncps that form *in vivo* and *in vitro* on the *in21* and *in25* constructs

Lanes 1 and 2, *in vivo* downstream borders, as determined on the *in21*- (lane 1) and *in25* (lane 2) carrying minichromosomes. The structure of the whole minichromosome is described in the text. PE of monomer-sized DNA was with primer # 1, as described in the Materials and methods section. The ncp borders are numbered between lanes 2 and 3 and are plotted in Figure 1. Lanes 3 and 4, borders of the *in vitro* reconstituted particles (see the Materials and methods section). Lane N, cleavages by MN on 146 bp fragments from the *Eco*RI–*Hin*dIII *in21* naked DNA (see the text). Lane P, elongation pausing on the *Eco*RI–*Hin*dIII *in21* fragment (see the text). Lane M, size markers (pBR322/*Msp*I). C and T, sequence lanes.

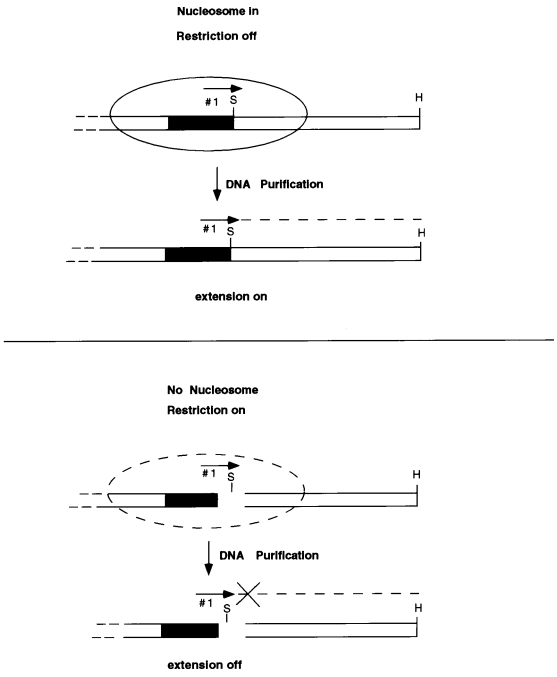

Figure 3 Schematic representation of the analysis of the *in vivo* accessibility to restriction enzymes

If the selected *Sau*3AI (S) restriction site is occupied by an ncp, restriction *in vivo* by externally supplemented enzyme is prevented. Upon purification of the *Eco*RI–*Hin*dIII fragment, the primer # 1 will hybridize and PE will proceed to the *Hin*dIII site (H) (upper panel). If on the contrary no ncp is present to protect the S site, restriction may occur, the primer will not be able to hybridize and PE will be prevented (lower panel). The necessary controls are described in the text.

fragment) with primer # 1 (lane P). Both controls show that the background of the system is negligible and confirm that the indicated fragments refer to real nucleosomal borders.

The result of the primer extension of monomeric DNA from *in vitro* reconstitution of the *in21 Eco*RI–*Hin*dIII fragment is shown in Figure 2 (lane 3). The corresponding ncp positions are diagrammed in Figure 1. The results are in good agreement with previous determinations: primer extension borders number 1–11 correspond to borders previously mapped by ExoIII number 6–10 and 12–17 (respectively; according to the original numbering in [26]). The only exception to the close match between the two techniques is the weakly detected ExoIII border number 11, which is completely undetectable by MN/PE. On the *in25* fragment (Figure 2, lane 4) the *in vitro* borders of ncps centred on the insertion (borders number 4–7) are much less intense, thus confirming a lower occupancy of the corresponding positions.

The picture that emerges from the *in vivo* analysis (Figure 2, lanes 1 and 2) on the same constructs is, in overall terms, qualitatively similar to that observed *in vitro*: multiple positions are occupied that form according to the same dominant rotational setting observed *in vitro*. Certain differences between *in vitro* and *in vivo* localization are also evident, especially so in the areas occupied by ncps number 2–4. The main difference is the *in vivo* absence of ncp number 4.

The intensity of the signals *in vivo* from the *in25* construct is lower than for *in21* (Figure 2; compare lane 1 with lane 2) (see also the experiments in the next section). This is an indication of lower affinity of *in25* for ncps *in vivo*, in agreement with the results of the measurements of the standard free energy of the binding affinity *in vitro* of a segment of the *in25* DNA relative to that of a corresponding segment of *in21*. An energy difference (ΔG°) of 432 cal (1.81 kJ)/mol was observed [26]. This difference in reconstitution energy indicates on a quantitative basis that disrupting rotational phasing disfavours nucleosome reconstitution.

In spite of these differences, the overall repetitive helically phased similarity between the two patterns is evident, thus

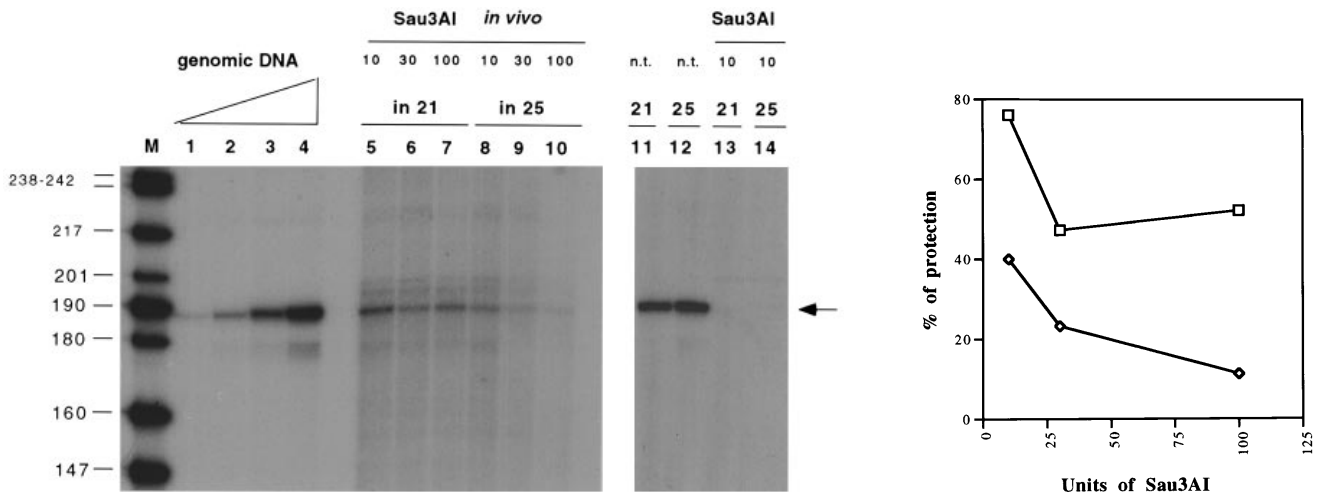

Figure 4 ***In vivo*** **accessibility to** ***Sau*****3AI restriction of** ***in21*****- or** ***in25*****-containing minichromosomes**

Left panel (lanes M and 1–14): lanes 1–4, 100, 200, 400 and 800 ng, respectively, of genomic DNA purified from *in21* minichromosome-containing permeabilized spheroplasts was restricted with *Hin*dIII and extended with primer # 1. Lanes 5–10, *in21*- (lanes 5–7) or *in25*- (lanes 8–10) containing permeabilized spheroplasts (10^6 in 0.4 ml) were treated with the amounts (units) of *Sau*3AI indicated at the top of each lane (1 h at 37 °C in 50 mM Tris/HCl, pH 7.9, 2 mM $MgCl_2$, 100 μg/ml nystatin, 50 mM NaCl and 1 mM PMSF). Genomic DNA was purified [26] and restricted with *Hin*dIII, and 400 ng/sample was extended with primer # 1. Lanes 11 and 12, spheroplasts were incubated without restriction, then processed as above. Lanes 13 and 14, samples were processed as for lanes 11 and 12, then, after the purification step, the DNA was extensively restricted with *Sau*3AI, thus preventing the PE reaction. M, size markers (nt). Right panel: the amount of PE product obtained after *in vivo Sau*3AI restriction, reported as a percentage of the reference unrestricted sample. Data for *in21* (□) are from lanes 5–7 compared with lane 11; data for *in25* (◇) are from lanes 8–10 compared with lane 12.

confirming that the yeast 5 S DNA sequence determines an *in vitro* and *in vivo* coherent, strongly defined rotational setting. Borders number 5, 6 and 8 are more prominent than others *in vivo* (Figure 2, lane 1), relative to the more homogeneous and complete pattern observed *in vitro* (lane 3). This difference could be due to topological effects caused by the closed structure of the minichromosome.

In conclusion, the relevant point here is that the analysis of the *in vivo* positions on the *in25*-containing minichromosome shows a strong exclusion effect by the 25 bp insertion on the nucleosomes centred on it (see the map in Figure 1). The decrease of the relative intensity of the borders of ncps number 5 and 6 is quite evident (Figure 2, lane 2).

In vivo accessibility to restriction enzymes

The *in vivo* mapping of ncp positions by PE of monomeric DNA shows that perturbing the dominant rotational setting by inserting two and a half turns in a central position has a destabilizing effect not only *in vitro* but also *in vivo*, leading to a lesser occupancy of the positions centred on the insertion. To confirm this lesser occupancy we have tested the *in vivo* accessibility of the inserted sequence to restriction enzymes and have developed a novel assay based on PE.

An initial problem is set by the necessity of distinguishing by Southern blot the plasmid-borne 5 S gene carrying the insertion from the endogenous chromosomal copies lacking it. This problem is solved by using, in the analytical PE assay described below, primer sequences complementary to DNA sequences artificially introduced into the 5 S rRNA. Thus, the assay reveals only the engineered plasmid-borne 5 S copies. The obvious control consisting of the comparison of the ncp patterns of the wild-type-5 S-containing plasmid versus the *in21*- and/or *in25*-containing plasmid cannot be performed, because it would be obscured by the signals produced by the chromosomal copies. The limitation intrinsic to this approach is that we do not analyse natural copies of the genes, and that we have to limit the validity of the conclusions to the comparison of the constructs bearing the 21 and 25 bp inserts. However, the comparison of the effects of these rotationally different constructs is precisely the goal of the present analysis.

The rationale of the experiment is illustrated in Figure 3: if the inserted DNA destabilizes the ncps centred on it, its sequence is expected to be less protected against *in vivo* restriction. In the assay, chromatin DNA is restricted at the *Sau*3AI site in permeabilized spheroplasts, then primer extended with primer # 1. A second restriction by *Hin*dIII, performed on the DNA after deproteinization, provides the end-point of the elongation. The PE is prevented by restriction by *Sau*3AI, so that, in the conditions in which the extension product is linearly proportional to the concentration of the template, its amount will be inversely proportional to the extent of restriction.

Figure 4 shows the results of this experiment. For the linearity controls, genomic DNA from yeast cells transformed with *in21*-containing plasmid was purified and restricted with *Hin*dIII. Extension of increasing, limiting amounts of this DNA with primer # 1 yielded increasing amounts of the expected 187-nt-long product (Figure 4, lanes 1–4). Ensuring that the assay is performed in linear PE conditions is essential. Given that PE efficiency depends on several factors, the major one being the nature of the DNA sequences involved, no additional internal control can be practically performed. Thus, particular care was taken in keeping the yields of the reactions under controlled and repeatable conditions. We have adopted the following precautions and verified several times the reproducibility of the results. For controls of yields and of inhibition by restriction, extensions of an equal amount of genomic DNA purified from strains transformed with *in21*- and *in25*-containing plasmids yielded comparable amounts of the product (Figure 4, lanes 11 and 12). Purified genomic DNA restricted with *Hin*dIII and

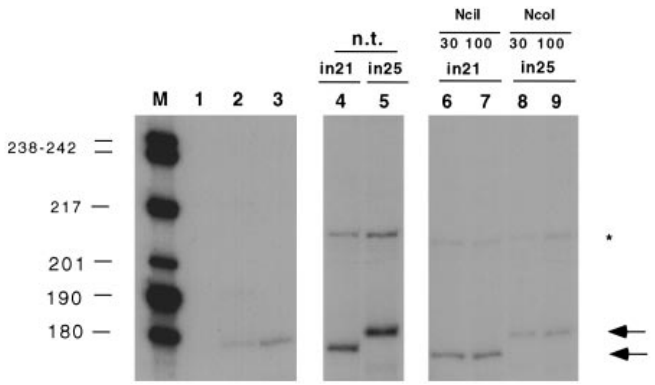

Figure 5 ***In vivo*** **accessibility of** ***in21*****- and** ***in25*****-containing minichromosomes to** ***Nci*****I and** ***Nco*****I restrictions**

Lanes 1–3, 100, 200 and 400 ng, respectively, of genomic DNA purified from *in25* minichromosome-containing permeabilized spheroplasts was restricted with *Eco*RI and extended with primer # 2. Lanes 4 and 5, *in21*- or *in25*-containing spheroplasts (10^6 in 0.4 ml) were incubated 1 h at 37 °C in the same restriction buffer as in the legend to Figure 4. Genomic DNA was purified and restricted with *Eco*RI, and 800 ng/sample was extended with primer # 2. Lanes 6–9, as above, on DNA restricted *in vivo* with *Nci*I (lanes 6 and 7; 30 and 100 units respectively) or *Nco*I (lanes 8 and 9; 30 and 100 units respectively), as indicated at the top of the appropriate lanes. The asterisk indicates an unspecific PE product. The arrows indicate the expected products, differing by 4 nt (173 and 177 nt for *in21* and *in25* respectively). M, size markers (nt).

*Sau*3AI did not yield any defined product (Figure 4, lanes 13 and 14). These controls ensure the quantitative reliability of the assay.

Restriction *in vivo* reduced the yield of PE products on both *in21*- (Figure 4, lanes 5–7) and *in25*-(lanes 8–10) carrying minichromosomes. However, the reduction is markedly more severe for *in25* than for *in21*. The percentage of minichromosomal substrate resistant to the restriction is quantified in Figure 4 (right panel).

A similar experiment was performed exploiting the restriction site *Nco*I, which cleaves inside the *in25* sequence and is converted into *Nci*I in the *in21* insertion [26]. The primer used in this case was # 2, and the secondary restriction was by *Eco*RI. The extension yields a product 4 nt longer for *in25* (177 compared with 173). Also in this case, restriction *in vivo* decreases the *in25* product more than the *in21* product (Figure 5, lanes 4–9). The amount of protected template is very similar to what was quantitatively determined in Figure 4 (right panel): $\approx 50\,\%$ for *in21* and $\approx 15\,\%$ for *in25*.

DNA sequences designed to position ncps *in vitro* have so far failed to do the same *in vivo*. A synthetic DNA sequence, composed of tandem repeats of a 20 bp sequence, denoted the 'TG sequence', was reported to associate with the histone octamer *in vitro* with an affinity 100-fold higher than nucleosomal DNA from biological sources [8]. However, similarly repeated TG sequences failed to position nucleosomes *in vivo* [11,30]. In addition, analysis of the *in vivo* nucleosomal structure of modified TG sequences designed to accommodate underwinding at the pseudodyad, and solve the *in vitro* versus *in vivo* discrepancy, has shown the absence of positioned complexes [31]. We report here that the natural sequence of the yeast 5 S rRNA gene provides a response to specific sequence modifications that is largely similar *in vitro* and *in vivo*. The 5 S rRNA gene system establishes itself as a system able to bypass the difficulties encountered by the *in vivo* use of completely artificial systems, thus facilitating the study of the rules governing nucleosome positioning *in vivo*.

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