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Nucleosome organization on *Kluyveromyces lactis* centromeric DNAs

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Abstract

The preferential assembly of specialized nucleosomes on budding yeast centromeres can be due either to the higher stability of specialized centromeric nucleosomes and/or to the lower stability of canonical centromeric nucleosomes with respect to bulk nucleosomes. We have evaluated the thermodynamic stability of canonical nucleosomes, assembled on *Kluyveromyces lactis* centromeric DNAs, with a competitive reconstitution assay and a theoretical method recently developed by us. The results, obtained by both methods, show that all five known centromeric DNAs from *K. lactis* are able to organize canonical nucleosomes, characterized by higher stability with respect those of bulk DNA. With 'footprinting' and theoretical prediction, based on sequence-dependent DNA elasticity, we have found that centromeric canonical nucleosomes are characterized by nucleosome dyad axis multiple positioning, rotationally phased. The isoenergetic nucleosome multiple positions are relevant in understanding the transition from canonical to specialized nucleosomes in interacting with centromere protein complexes. The satisfactory agreement between the results obtained from theoretical and experimental methods shows that sequence-dependent centromeric DNA elasticity has a main role in nucleosome thermodynamic stability and positioning.

Keywords: Sequence-dependent DNA elasticity; Nucleosome thermodynamic stability; Nucleosome multiple positioning; Centromere; *Kluyveromyces lactis*

1. Introduction

Centromeric DNA sequences in budding yeast, such as *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Kluyveromyces lactis* (*K. lactis*), have peculiar features shared by all chromosomes of the same organism (16 for *S. cerevisiae* and five out of six for *K. lactis*) and, to relevant extent, by the two different yeasts [1,2]. In both yeasts, centromeric DNAs are characterized by an AT rich (approx. 90%) central sequence, with a length of approxi-

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mately 80 bp in *S. cerevisiae* and 160 bp in *K. lactis*, flanked by two short tracts, required for specific centromeric proteins recognition.

A model of *S. cerevisiae* centromere proposed by Bloom et al. [3] and by Pluta et al. [4], supported by results obtained by Ortiz et al. [5] and by Fitzgerald-Hayes et al. [6], suggested that a modified nucleosome with the histone H3 variant Cse4p is an important element in centromere organization. The presence of a specialized nucleosome at centromeres seems now, established, but its relationships with canonical nucleosome and the process of transition from canonical to specialized centromeric nucleosome are, so far, not clarified, mainly for the reason that the histone H3 centromeric variant has not yet, been purified.

We have studied the canonical nucleosome thermodynamic stability on five centromeric DNAs from *K. lactis*, since the preferential organization of specialized nucleosome at centromeres can be due either to the higher stability of specialized centromeric nucleosome and/or to the lower stability of canonical centromeric nucleosome with respect to bulk nucleosome. We have used an in vitro approach based on a simplified model system, constituted by centromeric DNAs and purified histone octamer. This system can be used to study the ability of centromeric sequences to organize nucleosomes by evaluating their thermodynamic stability and positioning in comparison with bulk nucleosomes. We have chosen *K. lactis* rather than *S. cerevisiae* centromeric DNAs because their lengths, approximately 200 bp, allows us to consider the centromere sequence alone, without taking into account the centromere flanking regions. In this report we show that all centromeric DNAs of *K. lactis* are able to form nucleosomes thermodynamically more stable than bulk nucleosomes. Their stability, taking into account the good agreement with the theoretical analysis, carried out by the method, previously developed by us [7], is due mainly to sequence-dependent curvature and flexibility of centromeric DNAs. Nucleosome positioning on *K. lactis* chromosome 1 (KlCEN1) has been investigated by 'footprintings', to obtain information on canonical centromeric nucleosome positioning. We have found that KlCEN1 nucleosome is able to occupy multiple translational positions with the same rotational phase. The satisfactory correlation between the experimental and theoretical analysis of KlCEN1 nucleosome mapping indicates that both nucleosome positioning and nucleosome thermodynamic stability depends mainly on the sequence-dependent elastic properties of centromeric DNA. The theoretical analysis therefore allowed us to map nucleosome dyad axis positions on all *K. lactis* centromeric DNAs.

2. Materials and methods

2.1. DNAs

K. lactis centromeric DNAs of chromosome 1 (KlCEN1), chromosome 2 (KlCEN2), chromosome 3 (KlCEN3), chromosome 4 (KlCEN4) and chromosome 6 (KlCEN6) were derived, respectively, from the plasmids pKlCEN1-II.9, pKlCEN2-II.8, pKlCEN3-I.34, pKlCEN4-I.31 and pKlCEN6-I.2, that were obtained by A.A. Winkler and B.J.H. Zonnenveld whose characteristics are reported by Heus et al. [8]. The sequence features are schematized in Table 1. The sequence named TAND-1 (Travers Average Nucleosome DNA) was a gift of A. Travers and is shown in Table 1. All sequences were checked by dideoxy sequencing. DNA fragments used for polyacrylamide gel retardation measurements and for competitive reconstitution experiments were DNAs internally labeled by 30-cycle PCR amplification in the presence of [α-^{32}P]-dATP. Amplification products were purified on 5% native polyacrylamide gel.

KlCEN1 (216 bp) DNA fragment used for λ exonuclease, DNase I and hydroxyl radical has been subcloned in the EcoRI–BamHI site of pUC18 (pKlCEN1). The DNA fragment (KlCEN1) was radiolabeled at only one strand as follows: plasmid containing the centromeric sequence was linearized with a first restriction enzyme (EcoRI or BamHI), 3′-end-labelled by filling in with [α-^{32}P]-dATP and sequenase enzyme, and finally digested with the second restriction enzyme. The labeled fragments were separated on 5% (w/v) polyacrylamide gel elec-

Table 1
Kluyveromyces lactis centromeric DNAs sequences

	KlCDEI	KlCDEII		KlCDEIII	
	⇓	⇓	⇓		
KlCEN1 (216 bp)	P-GCACGTGA	[161 bp (87%AT)]		TGCTTTATGTTTCCGAAAAT-TATTTT-P′	
	⇓	⇓	⇓		
KlCEN2 (219 bp)	P-ATCACGTGA	[164 bp (91%AT)]		CAAAGTTTGTTTCCGAAAAT-TAAAAT-P′	
	⇓	⇓	⇓		
KlCEN3 (218 bp)	P-ATCATGTGA	[163 bp (89%AT)]		AAATTTTAGTTTCCGAAAAT-TAATTT-P′	
	⇓	⇓	⇓		
KlCEN4 (219 bp)	P-ATCACGTGC	[164 bp (86%AT)]		TTTAATACAGTTCCGAAAATAAA-TAT-P′	
	⇓	⇓	⇓		
KlCEN6 (218 bp)	P-TGCACGTGA	[163 bp (91%AT)]		TGTTTTATTGTTCCGAAAA-TAAAAAT-P′	
TAND-1 (159 bp)	GATCCCCTTT CTTTGGATCA AGCCTTACTA GAGGGAATT	TTAATTGCTC CATGAGCTAA CCTTTACATT	AGCTCTAGAG TAAAGTGCTT AACTCCTTGC	GTGAAATGTC CCTGCTAAAC TTTCTGTCTA	TCACAGGATA TGAGAGGAAC AAAATGTAGG

Centromeric sequences are those reported in Keith and Fitzgerald-Hayes [6]. KlCDEI, KlCDEII and KlCDEIII indicate the centromeric proteins target sites. P and P′ are primers used to recover by PCR the fragments used for competitive reconstitution and cloning. TAND1 sequence was used as reference.

trophoresis, recovered from the gel and then purified by phenol extraction and ethanol precipitation.

2.2. Nucleosome competitive reconstitution assay

The procedure used for competitive reconstitution was that of Shrader and Crothers [9,10] with minor modifications. Three micrograms of H5-depleted poly-nucleosomes, obtained from chicken erythrocytes, were mixed with 30 ng of radiolabeled centromeric DNA and various amounts of sonicated calf thymus DNA as competitor in 0.9 M NaCl, 10 mM Tris–HCl pH 8, 1 mM EDTA pH 8, 0.1% Nonidet P40, in a volume of 6 μl. After incubation at room temperature for 30 min, the salt concentration was lowered to 50 mM NaCl, step-by-step with additions of the same buffer without NaCl. Samples were resolved on 5% polyacrylamide gel in 0.5X TBE buffer (90 mM Tris–Borate, 2 mM EDTA, pH 8.3). The relative quantities of reconstituted and free DNA were assayed by scanning dried gels with Instant Imager (Packard). The free energy for a given centromeric sequence (CEN) was calculated from the equation

$$\Delta\Delta G\ (\mathrm{CEN}) = RT\ln\left[\alpha\ (\mathrm{TAND\text{-}1})\right] - RT\ln\left[\alpha\ (\mathrm{CEN})\right] \quad (1)$$

where α(TAND-1) is the ratio of labeled reconstituted nucleosome to labeled free DNA for the reference sequence TAND-1, and α(CEN) is the analogous ratio for centromeric sequences. Each fragment was reconstituted at least in three separate experiments and the results obtained were averaged.

2.3. λ Exonuclease footprinting assay

Reconstituted end-labeled DNA after desalting with micropure separators was digested in 2.5 mM $MgCl_2$ with 25 U/μl of λ Exonuclease at 37 °C for 20, 40 and 60 min [11]. The reaction was stopped by adding an equal volume of 20 mM EDTA and 0.2% SDS. Samples were phenol extracted and analyzed on 6% polyacrylamide-urea

sequencing gels. Gels were autoradiographed and autoradiographs were scanned with a BioRad GS-670 imaging densitometer.

2.4. DNase I footprinting assay

DNase I footprinting of reconstituted nucleosome was performed in parallel with free DNA samples dissolved in the same final buffer. To avoid misinterpretations due to the presence of free DNA, nucleosome samples, after cleavage with DNase I, were run on a preparative 5% polyacrylamide gel. The nucleosome band was excised and then purified.

DNase I digestion was carried out in 70 μl containing 5 mM $MgCl_2$ for 1 min at room temperature with 0.2 U/ml of enzyme for naked and 2–10 U/ml for reconstituted DNA. The reaction was stopped by adding the same volume of 20 mM EDTA [12]. Samples were then phenol extracted and precipitated with ethanol. Separation on 6% denaturing polyacrylamide sequencing gels and exposure were conducted as described above.

2.5. Hydroxyl radicals footprinting assay

As for DNase I, hydroxyl radical footprinting assay was performed in parallel with free DNA samples, dissolved in the same final buffer. To avoid misinterpretations due to the presence of free DNA, nucleosome samples, after reaction with hydroxylradicals, were run on a preparative 5% polyacrylamide gel. The nucleosome band was excised and then purified. The reaction cutting with hydroxyl radicals, described by Rossetti et al. [13], was carried out in 50 μl of 1 mM ascorbic acid, 0.03% H_2O_2, 50 mM $(NH_4)_2Fe(SO_4)_2$ and 100 mM EDTA for 2 min at room temperature. The concentration of reagents used for naked DNA was 10 times lower than that used for reconstituted DNA. The reaction was stopped by adding an equal volume of 140 mM Thiourea and 140 mM EDTA. Samples were then phenol extracted and precipitated with ethanol. Separation on 6% denaturing polyacrylamide sequencing gels and exposure were conducted as described above.

3. Results

3.1. Polyacrylamide gel electrophoretic mobility measurements and theoretical analysis show that K. lactis centromeric DNAs are slightly curved

The curvature of DNA fragments is usually assayed by measuring their electrophoretic mobility on polyacrylamide gel. In fact, the retardation ratio, *R*, which is the ratio between the measured and real molecular weight of DNA, can be considered as a measure of DNA curvature when the gel concentration is sufficiently high [14]. By this method we analyzed the mobility of all centromeric DNAs from *K. lactis*. To this end we amplified the centromeric sequences from the CDE I box to the CDE III box, obtaining in all cases a 200-bp-long centromeric DNA fragment, namely KlCEN1, KlCEN2, KlCEN3, KlCEN4 and KlCEN6, which refer, respectively, to chromosomes 1, 2, 3, 4 and 6 (see Table 1). These fragments, carefully purified, were assayed for their mobility on 8% polyacrylamide gel, using, as molecular weight marker, pUC 18 cleaved with Hpa II. All centromeric DNA fragments migrated significantly more slowly than a fragment of the same length of the molecular weight marker (lanes 1, 2, 3, 4 and 5 in Fig. 1a). The very high AT content of *K. lactis* centromeric DNAs (see Table 1) suggests that these DNAs should be highly curved. The electrophoretic analysis, instead, showed that the retardation ratios are in the 1.05–1.20 range, thereby indicating a slight global curvature (see Table 2). We measured the gel electrophoretic mobility of centromeric DNAs by decreasing the temperature and adding the polyamine spermine, a poly-cation that increases DNA curvature [15]. The retardation ratio of all centromeric DNAs slightly decreased as the temperature was lowered to 4 °C (not shown), in agreement with results recently obtained for all *S. cerevisiae* centromeric DNAs by Bechert et al. [16]. This behavior, which is the opposite to that presented by most sequences, can be attributed to the high flexibility of AT-rich sequence not phased [7]. By contrast, spermine

slightly increased DNA curvature as shown in Fig. 1b and Table 2. To connect the electrophoretic behavior of centromeric DNAs to the DNA curvature and flexibility, the experimentally measured retardation ratios were compared with those theoretically calculated [7] as showed in Table 2. The largest difference between experimental and theoretical retardation ratios is +0.03, except for KlCEN4, where the difference is −0.10. This could be explained by taking into account that the retardation ratio depends on the temperature in a sequence-dependent mode [16]. This suggestion seems feasible on account of KlCEN4 different behavior compared with the other investigated centromeric DNA samples in electrophoresis with spermine (Table 2). KlCEN4 appears the only centromeric DNA unaffected by the polyamine presence in solution. Experimental as well as theoretical retardation low value ratios suggest that *K. lactis* centromeric DNAs should have a lower global curvature than a number of sequences with a comparable high AT content (approx. 90%) so far investigated, such as some *Crithidia fasciculata* and the *Leishmania tarentolae* kinetoplast DNA tracts [17,18].

Table 2
Retardation ratios (R) of *K. lactis* centromeric DNA fragments

DNA	R_{exp} (20 °C)	R_{exp} (20 °C/SPM)	R_{TH}
KlCEN1	1.19	1.21	1.18
KlCEN2	1.10	1.16	1.07
KlCEN3	1.11	1.20	1.08
KlCEN4	1.12	1.11	1.22
KlCEN6	1.09	1.12	1.07

Eight percent gel electrophoretic mobilities reported as retardation ratios of *K. lactis* for five centromeric DNAs at 20 °C in 1×TBE buffer (R_{exp}/20 °C) and in the same buffer with 0.01 M spermin (R_{exp}/20 °C/SPM). The average error, derived from at least three independent measurements, is ±0.03. The table also shows retardation ratios calculated by theoretical analysis.

3.2. Evaluation of experimental and theoretical free energies of nucleosome formation shows that all K. lactis centromeric DNAs are intrinsically able to organize canonical nucleosomes, which are thermodynamically, more stable than bulk nucleosomes

To measure the free energy of nucleosome formation on centromeric DNAs, we used the com-

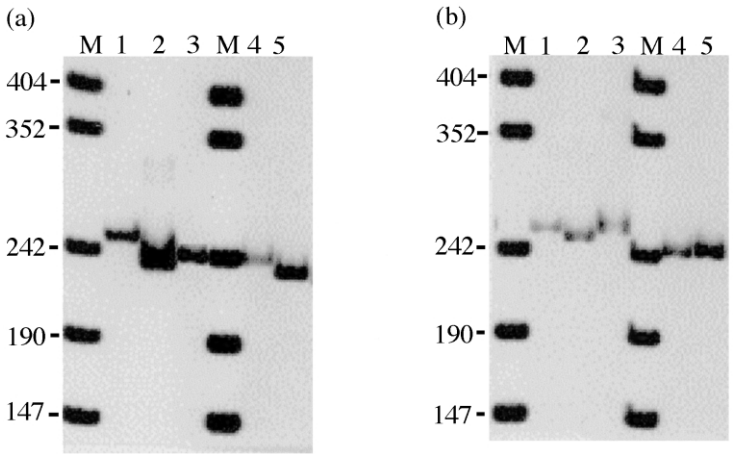

Fig. 1. Electrophoretic retardation assay. Electrophoretic retardation assay of five KlCEN1 centromeric DNA fragments, reported in Table 1, on an 8% native polyacrylamide gel at 20 °C in TBE buffer 1X (a), and as in (a) with 0.01 M spermin added (b). M is pUC18 cleaved with the restriction enzyme HpaII (lanes 1 and 5). Lanes 2, 3, 4, 6 and 7 correspond, respectively, to KlCEN1, KlCEN2, KlCEN3, KlCEN4 and KlCEN6 DNA fragments.

petitive reconstitution assay, in which a competition for a limited number of histone octamer is established between radiolabeled centromeric DNA and unlabeled heterogeneous sequence DNA. The fraction of labeled DNA present in the nucleosomal band depends on its intrinsic ability to form nucleosome compared with bulk DNA. One of the main problems in evaluating the free energies of nucleosome formation on several sequences is the choice of reference DNA. In most studies average nucleosomal DNA has been adopted as standard DNA. The use of this type of DNA has the advantage of a clear biological meaning but its heterogeneity could give rise to a significant error. We used as standard DNA a unique sequence from the set of 177 nucleosomal sequences cloned by Satchwell et al. [19]. Most of these sequences should have an affinity for histone octamer practically equal to that of average nucleosomal DNA. However, we tested by competitive reconstitution, some of these nucleosomal sequences [11] to be sure we chose a standard DNA with practically the same free energy of nucleosome formation as average nucleosomal DNA.

The sequence of this nucleosomal DNA, which we named TAND-1 (Travers Average Nucleosomal DNA) and which has a $\Delta\Delta G$ of approximately -0.20 Kcal/mol of nucleosome with respect to bulk nucleosome is shown in Table 1. With TAND-1 as standard, we again measured the free energy values of nucleosome formed on the previously investigated centromeric sequence [20] as well as the free energy of nucleosome formation on other known *K. lactis* centromeric DNAs, namely KlCEN2, KlCEN3, KlCEN4 and KlCEN6 (Fig. 2a). The reported values are from at least three independent experiments carried out with three DNA/nucleosome ratios for each experiment and are shown in Fig. 2b. The results clearly indicate that all *K. lactis* centromeric DNAs organize more stable nucleosomes than the TAND1 sequence, which we assume is adequate to represent bulk nucleosome.

We used the recently developed theoretical model [21,22] to clarify the respective roles of sequence-dependent DNA elasticity, and of the local specific interactions between histone octamer and DNA in determining the free energy of nucleosome formation

The outline of the model can be summarized as follows. The free energy to transform the DNA intrinsic superstructure into a nucleosomal structure was predicted by adopting a simple first order elasticity theory. The DNA intrinsic superstructure was calculated using the same roll and tilt angles we derived before and that successfully worked in predicting gel electrophoretic retardation [9]. The DNA flexibility was derived from standard torsional and bending force constants multiplied by the empirical normalized melting temperature for each dinucleotide step [21,22] to represent the differential flexibility along the sequence. If $\Delta G(k)$ represents the nucleosome reconstitution free energy difference from a standard nucleosome of the kth DNA tract 144 bp (L) along a sequence, the free energy of nucleosome pertinent to the whole DNA chain, with N bp, will be:

$$\Delta G/RT = -\ln \sum_{K=L/2}^{N-L/2} \exp\left(-\Delta G(k)/RT\right) \qquad (2)$$

considering the global reaction as a sum of parallel reactions, the different equilibrium constants, pertinent to all possible nucleosome positions, sum up.

Using a statistical thermodynamic approach [17], we evaluate $\Delta G(k)$.

$$\Delta G(k)/RT = \Delta E^0(k)/RT - 3/2L\ln\left\langle T/T^*\right\rangle + b\left\langle T/T^*\right\rangle A_n A_f^0/(LRT) - \ln J_0\left(ib\left\langle T/T^*\right\rangle A_n A_f^0/(LRT)\right) \qquad (3)$$

where $\Delta E^0(k)$ is the minimum elasticity energy required to distort the kth 144 bp tract (L) in nucleosomal form; b is the apparent isotropic bending force constant; T is the dinucleotide empirical melting temperature as evaluated by Gotoh and Takashira [23] and T^* the relative mean value. The T/T^* ratio modulates the force constants along the sequence, producing a sequence-dependent flexibility. A_f^0 represents the effective curvature, namely the Fourier term of the free DNA curvature function, which coherently contributes to the nucleosomal structure, whereas fixing A_n is a sufficient condition to ensure that DNA assumes a nucleosome like form. J_0 is the zero order Bessel function.

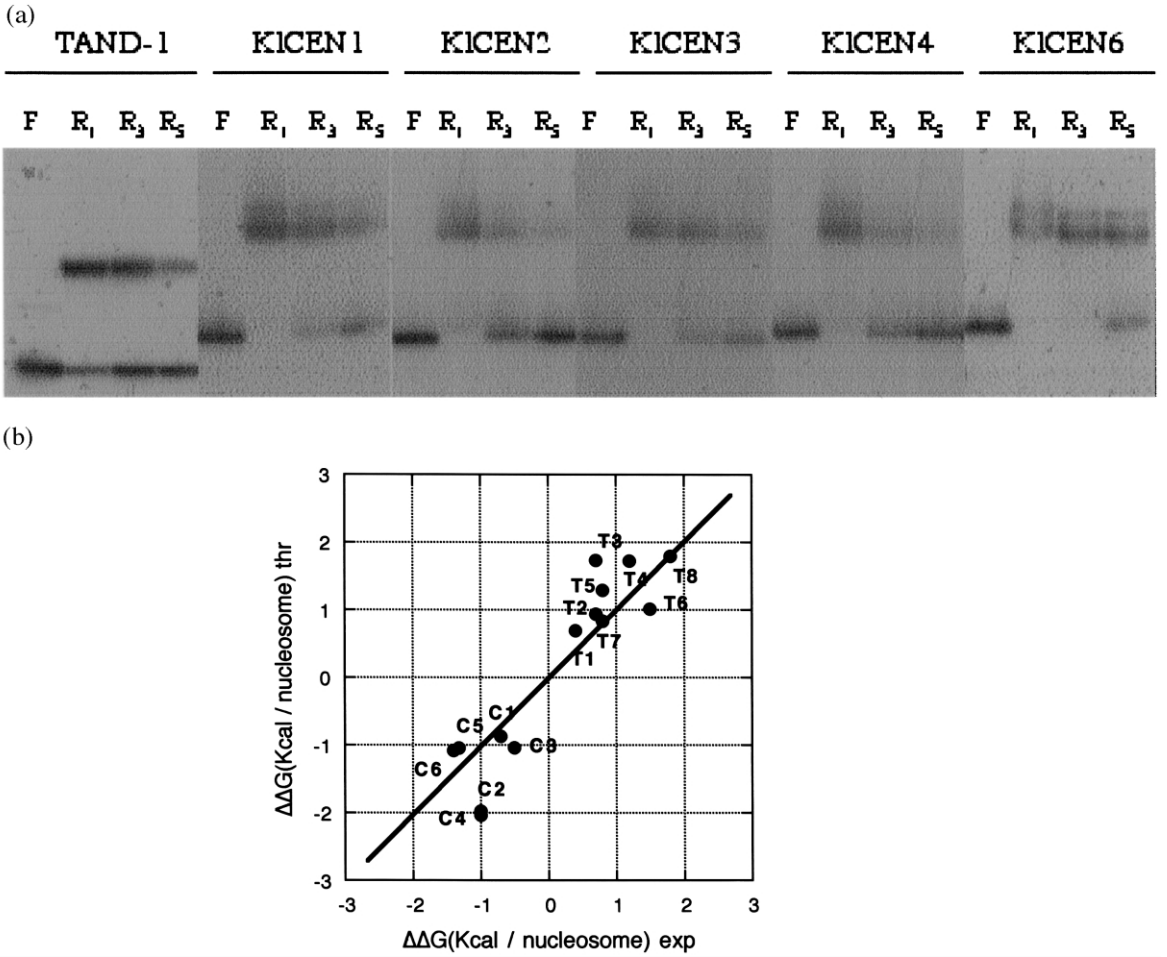

Fig. 2. (a) Competitive nucleosome reconstitution assay. An example of nucleosome competitive reconstitution experiment of five KlCEN1 centromeric DNA fragments and TAND-1 reported in Table 1. F is naked DNA. R is nucleosome core reconstituted on DNA with 1 (R_1), 3 (R_3) and 5 (R_5) μg of sonicated calf thymus DNA as competitor. (b) Comparison between experimental and theoretical free energy of nucleosome formation. The symbols indicate DNA sequences as follows: KlCEN1 (C1); KlCEN2 (C2); KlCEN3 (C3); KlCEN4 (C4); KlCEN6 (C5); and SCEN6 (C6). For the sake of comparison eight Telomeric sequences previously investigated by us [13] have been also reported: T1 (195 bp) and T2 (236 bp) are from Arabidopsis; T3 (171 bp) is from Bombyx; T4 (192 bp) and T5 (254 bp) are Human; T6 (198 bp) and T7 (216) are from Chlamydomonas; and T8 (185 bp) is from Tetrahymena.

The curvature function for a defined DNA sequence was calculated using the roll, tilt and twist angles used to satisfactorily calculate other properties dependent on DNA curvature such as gel electrophoresis mobility. The values of bending and twisting force constants were derived from a DNA persistence length of 450 Å and a torsional rigidity of 2.1×10^{-19} dyne cm^{-1} [24].

The results, shown in Fig. 2b, illustrate the comparison between theoretical and experimental free energy values of nucleosome formation. The value of $\Delta\Delta G=0$ Kcal/nucleosome corresponds to the center of the Gaussian, which represents the free energy values of the Satchwell/Travers nucleosomes ensemble (to be published). All nucleosomes reconstitute, on centromeric *K. lactis* DNA,

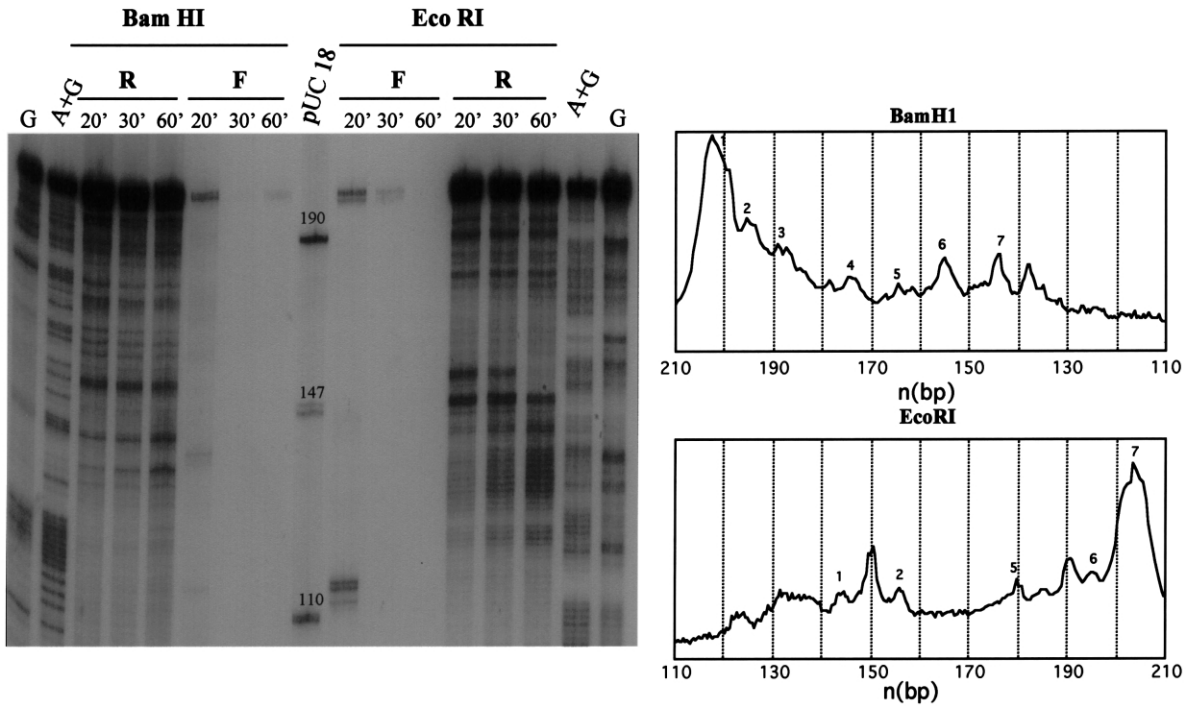

Fig. 3. λ Exonuclease footprinting assay. Left: λ exonuclease footprinting of nucleosome reconstituted onto KlCEN1 3′ end-labeled at Bam H1 and EcoR1 site centromeric DNA. M is pUC18 cleaved with Msp1. A+G is a Maxam–Gilbert sequencing reaction for purines. G is a Maxam–Gilbert sequencing reaction for guanine. F is naked DNA. R is nucleosome complex. Right: densitometric profiles of reported footprintings. In all cases bands corresponding to the borders of the same nucleosome have been labeled with the same number.

with a formation free energy lower than that of bulk nucleosomes, so they can be considered as more stable than average nucleosomes. The agreement between the theoretical and experimental values is satisfactory, showing that sequence-dependent DNA elastic properties are the main determinants in modulating the association constants values between histone octamer and DNA sequences. As previously reported [7], the high flexibility of centromeric DNA_S, due to their large AT content has a dual role on nucleosome thermodynamic stability. In fact, DNA flexibility decreases the distortion energy for nucleosome formation, but increases the entropy difference between the flexible naked DNA and the final rather rigid nucleosomal structure. The balance of the two contributions, because of some degree of curvature, favors the nucleosome formation in the case of centromeric DNA_S. For the sake of comparison, in Fig. 2b the values of $\Delta\Delta G$ for telomeric sequences, previously measured by us [11] are also reported. Unlike centromeric nucleosomes, all telomeric nucleosomes have a lower stability respect to average nucleosomes.

3.3. Mapping nucleosome positions on KlCEN1 DNA by λ exonuclease assay shows nucleosome multiple translational positioning with the same rotational phase

Nucleosome positioning on KlCEN1 DNA was investigated by exonuclease digestion. Many studies show that cleavage with Exo III, of nucleosome reconstituted on different sequences, allows an assay of nucleosome translational positioning [25,26]. In this study, we used λ exonuclease to

overcome the problem of exonuclease III (Exo III) sequence specificity. In fact, A and T residues, mostly present in centromeric DNAs, are released by Exo III at an intermediate rate, compared with C-residues and G-residues [27]. λ exonuclease degrades double-stranded DNA starting from the 5′ end of the molecule. The rate of digestion by λ exonuclease is highly processive and independent of DNA base composition [28]. The borders of the histone octamer complex block the progression of the enzyme and λ exonuclease digestion should therefore signal the presence of major nucleosome positions. Fig. 3 shows λ exonuclease footprinting of naked and nucleosome reconstituted KlCEN1 3′-end labeled on either strand, at increasing reaction times. Already after 20 min of digestion the band pattern of naked DNA disappears, while the positions and intensities of λ exonuclease stops do not change with increasing time, indicating that all mononucleosome main positions have been detected. The band patterns appear complex, indicating that nucleosomes have more than one preferential location on the sequences considered, and that a number of differently populated sets of molecules with one well positioned nucleosome are present. In such controlled experimental conditions, λ exonuclease allows the identification of nucleosome dyad axis positions [11]. In order to localize nucleosome stops on the DNA sequences, the band patterns were analyzed as densitometric tracings in both centromeric DNA fragments. The nucleosome borders identified on the EcoRI end-labeled strand correspond to the nucleosome borders found on the BamHI end-labeled strand that is 146 ± 2 bp away. Seven main positions, assigned by taking into consideration λ exonuclease stops from both directions, are detectable on reconstituted samples and give rise to bands spaced approximately every 10 bp. The digestion pattern phases, equal to the B-DNA helical repeat, suggests that KlCEN1 centromeric nucleosome adopts a multiple translational positioning with the same rotational phase. It is worth noting that recently, it has been shown that the λ exonuclease results are in good agreement with those obtained by the site-directed cleavage method [29].

3.4. DNase I and hydroxy radical footprintings of nucleosome reconstituted on KlCEN1 centromeric DNA show that the centromeric nucleosome is rotationally phased

In order to measure the nucleosome dyad axis positions more precisely; we used DNase I footprinting of nucleosome reconstituted on KlCEN1 DNA.

It is well known that DNase I footprinting provides information on the helical periodicity of DNA-protein interactions [30]. Favored positions of cleavage within the nucleosome core DNA lie at intervals of approximately 10 nucleotides, corresponding to the points where the minor groove faces outward. When the DNA sequence is rotationally positioned in relation to the histone octamer, the same DNA face will always be exposed on the outside; therefore, DNase I cleavage will generate a 10 bp ladder. DNAs without a preferred rotational positioning or with several alternative frames of rotational positioning will not show a clear 10 bp cleavage periodicity or will even lack a nucleosome-specific DNase I pattern. The DNase I footprintings of KlCEN1 are shown in Fig. 4. Both in naked DNA and in nucleosome, DNase I digestion patterns revealed a strong sequence-dependent cleavage specificity [31] due to the extraordinarily high AT content in the sequence (approx. 90%). In fact, AT-rich sequences are characterized by a narrow minor groove that is inaccessible to DNase I. As in the case of *Crithidia fasciculata*, a strongly curved DNA previously studied by us [12,32], the DNase I digestion pattern is practically equal in both naked and nucleosome reconstituted KlCEN1DNA. Both patterns show a 10 bp periodicity, which allowed obtaining, for nucleosome reconstituted samples, the nucleosome rotational phasing [33]. Since DNase I digestion patterns have revealed a strong sequence-dependent cleavage specificity, we carried out similar digestion experiments using hydroxyl radicals. Hydroxyl radical cleaves DNA by abstracting a hydrogen atom from the deoxyribose sugar along the DNA backbone, moreover, it is exceedingly short lived and reactive so attack sites on DNA molecule surface, without sequence,

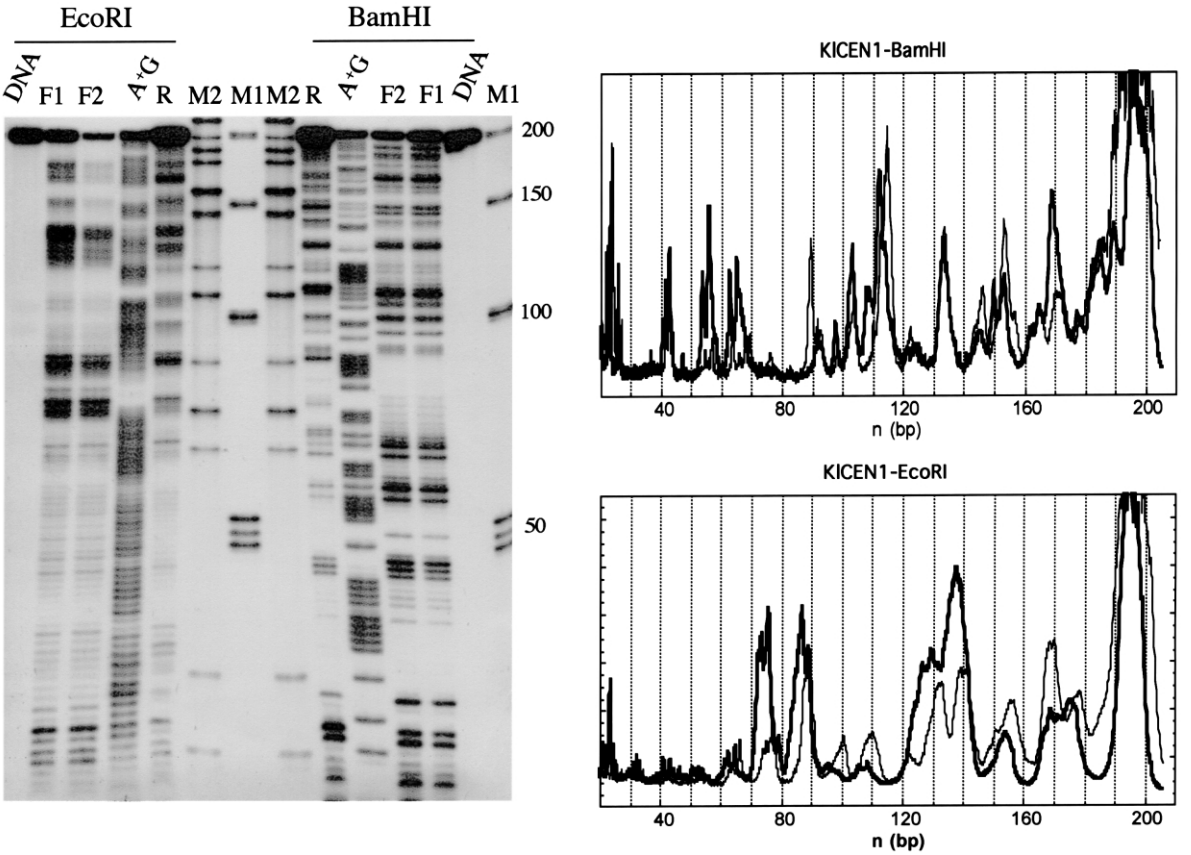

Fig. 4. DNase I footprinting assay. Left: DNase I footprinting of nucleosome reconstituted on KlCEN1 3′ end-labelled at Bam H1 and EcoR1 site centromeric DNA. A+G is a Maxam–Gilbert sequencing reaction for purines. M1 is a 50 bp ladder. M2 is pUC18 digested with Msp I. F is naked DNA cleaved with two different amounts of enzyme (F1 and F2). R is nucleosome complex. Right: densitometric profiles of reported footprints. In each panel the thick profile refers to free DNA and the thin profile to the nucleosome complex.

or base dependence, in the cleavage reaction [34,35]. The small size and the low sequence specificity of the hydroxyl radicals compared with DNase I, make this probe a widespread tool to measure DNA periodicity on the nucleosome. The hydroxyl radical footprintings of naked and nucleosome reconstituted KlCEN1 DNA are shown in Fig. 5. The hydroxyl radical cleavage of the centromeric nucleosome clearly shows the same 10 bp periodicity as canonical nucleosomal DNA, thereby confirming that KlCEN1 nucleosome is rotationally phased, and allowing the nucleosome dyad axis positions to be mapped.

3.5. KlCEN1 nucleosome multiple translational positioning from theoretical mapping correlates satisfactorily with experimental mapping

The evaluation of the free energy of nucleosome formation from the DNA sequence contains the evaluation of the modulation of the free energy along the sequence. The global value is obtained by summing up all the free energy contributions along the sequence (see Eq. (2)). Fig. 6 shows the theoretical nucleosome mapping along KlCEN1.

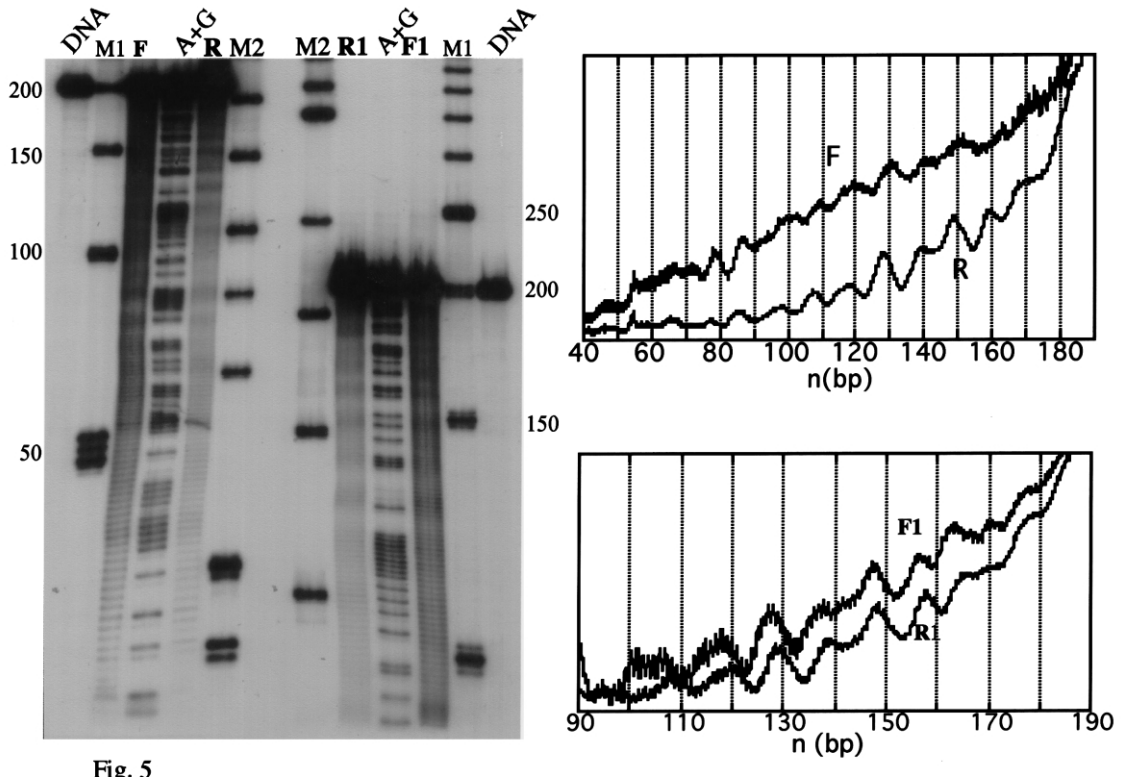

Fig. 5

Fig. 5. Hydroxyl radicals footprinting assay. Left: hydroxyl radicals footprinting of nucleosome reconstituted on KlCEN1 3′ end-labelled at Bam H1 site centromeric DNA. A + G is a Maxam–Gilbert sequencing reaction for purines. M1 is a 50 bp ladder. M2 is pUC18 digested with Msp I. F and F1 are naked DNA cleaved by hydroxyl radicals. R and R1 are the nucleosome complex cleaved by hydroxyl radicals. All samples were loaded twice at 1.5 h intervals. The set of lanes with F1 and R1 correspond to the first loading.

The theoretical analysis predicts nucleosome dyad axis multiple positions with a periodicity equal to 10. The values of free energy corresponding to the minima are all equal and can be interpreted as a degeneration of multiple translational nucleosome positions on KlCEN1. In principle, these findings suggest an equivalent occupancy of the different minima along the DNA sequence in vitro, although in vivo the flanking DNA sequences could influence nucleosome positioning. The experimental nucleosome dyad axis positions derived from λ exonuclease analysis correlate very well with the theoretical mapping (Fig. 7). Furthermore, a number of nucleosome dyad axis positions, compatible with the rotational orientation of the centromeric nucleosome, can be derived from DNase I and hydroxyl radicals footprintings which both correlate satisfactory with theoretical prediction too.

4. Discussion

We have recently developed a theoretical model, based on a statistical thermodynamic approach, which evaluates the influence of sequence-dependent DNA elasticity in determining the free energy of nucleosome formation in comparison between different DNAs. This model is based on the hypothesis that nucleosome stability depends on the elastic energy of bending and twisting to transform the DNA intrinsic superstructure into the nucleosomal structure. It has allowed us to predict the free energies of nucleosome formation in the case of approximately 60 different synthetic

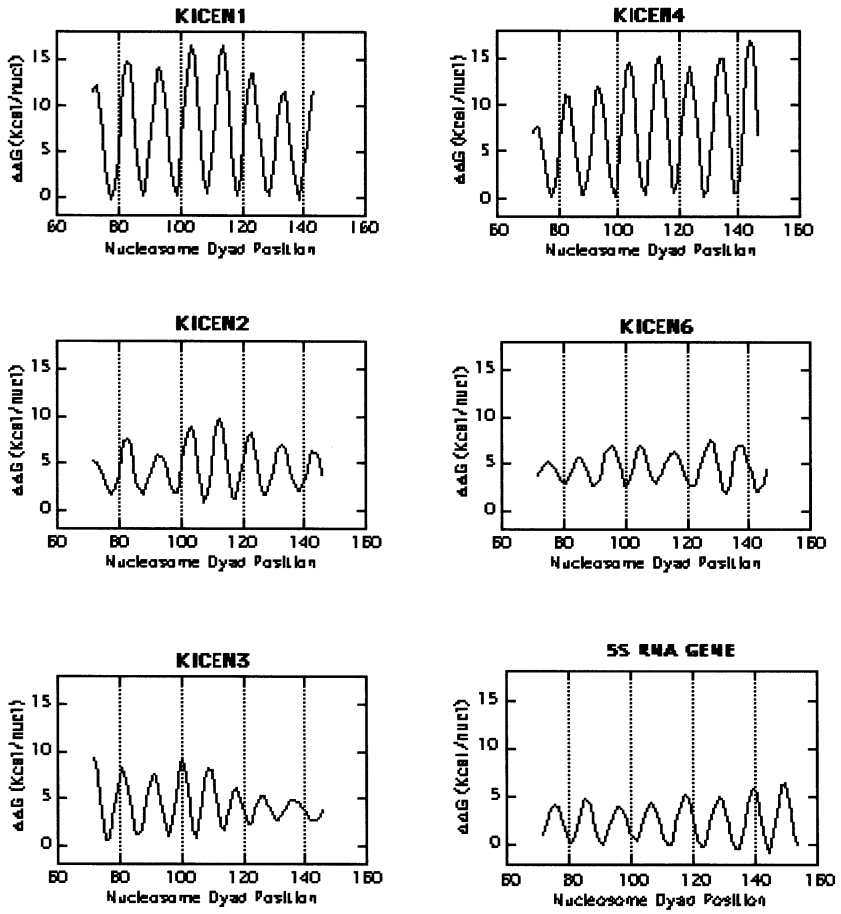

Fig. 6. Theoretical nucleosome dyad axis positioning. Theoretical nucleosome dyad axis positioning along five centromeric DNAs: KlCEN1; KlCEN2; KlCEN3; KlCEN4; and KlCEN6. For the sake of comparison theoretical nucleosome dyad axis positioning along the 5S RNA gene is reported on the bottom right.

or biological DNA sequences in fairly good agreement with the experimental measurements, carried out by competitive reconstitution assay [21,22]. How does this model apply in the case of *K. lactis* centromeric nucleosomes, all characterized by low curvature? The results reported in Fig. 2a,b show that nucleosomes organized on *K. lactis* centromeric DNAs, and on *S. cerevisiae* chromosome 6 centromeric DNA, are in all cases more stable than bulk nucleosome. For sake of comparison, we have reported also the theoretical and experimental free energies of nucleosome formed on telomeric DNAs, previously investigated by us [11]. We found that telomeric nucleosomes are characterized by the lower association constant between histone octamer and DNA's biological tracts, so far found. The biological relevance of these different features of centromeric and telomeric nucleosomes, at least in the case of the sequences investigated by us, is at the moment, under investigation. The satisfactory correlation between experimental and theoretical results, shows that sequence-dependent DNA curvature and elasticity play the main role in determining the free energy cost of nucleosome formation and that local chemical signals are relevant as a second order factor in nucleosome thermodynamic stability. In this respect, it is worth noting that recently it has been shown that the

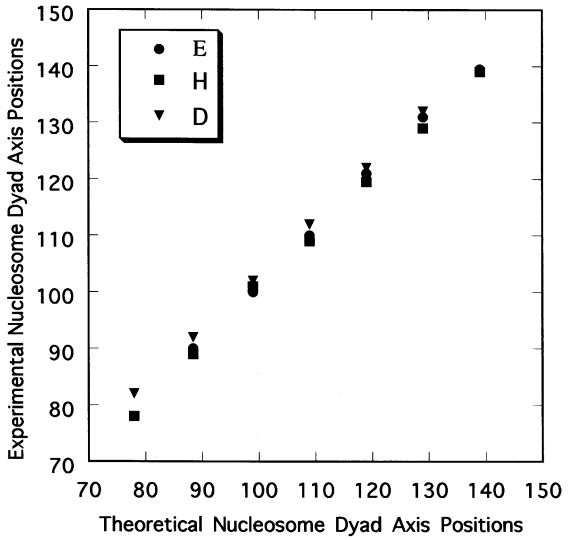

Fig. 7. Comparison between experimental and theoretical nucleosome dyad axis positions. Comparison between nucleosome dyad axis positions as derived from λ exonuclease (E), DNase I (D), hydroxyl radicals (H) footprintings and theoretical prediction.

nucleosome structure solved by Luger et al. [36] is not perturbed if the same DNA sequence is assembled as a nucleosome substituting the canonical histone octamer with the yeast histone octamer, [37] or the histone variant H2AZ [38].

The finding that *K. lactis* centromeric DNAs, as well as the previously investigated *S. cerevisiae* chromosome 6 centromeric DNA [20], are all intrinsically able to organize thermodynamically more stable nucleosomes than bulk DNA, suggests that centromeric canonical nucleosome could be substituted by specialized centromeric nucleosome, mainly on account of specific interactions with centromeric proteins. It is worth noting that this hypothesis could be directly assayed when the histone H3 variant, Cs4p, and the centromeric proteins interacting with its NH_2 tail will be purified. Recent results, obtained for the mammal histone H3 variant CENPA, seem encouraging in this respect [39,40]. Taking into account the biological function of the centromere, it seems reasonable to suppose that the centromeric canonical nucleosome cannot occupy a unique position; in fact, a mobile nucleosome appears more suitable for its transition to a specialized centromeric nucleosome.

The possibility to evaluate the nucleosome positioning features of the other *K. lactis* centromeric sequences appears relevant in developing a suitable model. We have taken advantage of the very satisfactory correlation between experimental and theoretical nucleosome mapping (see Fig. 7) found in the case of KlCEN1 nucleosome, to carry out the theoretical analysis of the other four *K. lactis* centromeric nucleosome positioning. The results are shown in Fig. 6, compared with those obtained for the 5S RNA gene, that is a sequence characterized by multiple nucleosome positioning rotationally phased; in this case, the binding of histone H1 or TFIIIA is ruled by nucleosome positioning, as shown by Panetta et al. [41].

It is tempting to suggest a similar mechanism in the transition from canonical to specialized centromeric nucleosomes, where the binding of a centromere protein complex to the specialized centromeric nucleosome should require defined nucleosome positioning [42,43].

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