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DNA superstructural features and nucleosomal organization of the two centromeres of *Kluyveromyces lactis* chromosome 1 and *Saccharomyces cerevisiae* chromosome 6

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Abstract Superstructural features of the *Kluyveromyces lactis* chromosome 1 (KlCEN1) and of the *Saccharomyces cerevisiae* chromosome 6 (SCEN6) centromeric DNAs were evaluated using a theoretical method, developed by our group, and experimentally measured by gel electrophoretic retardation. Both methods show that, in spite of the remarkable AT richness of the two centromeric sequences, their curvature is not very high. However the peculiar sequence features of the two centromeres allow to organize highly stable nucleosomes, with a free energy about that of the nucleosome formed on the 5S RNA gene. The good agreement between experimental and theoretical evaluation of nucleosome free energies as well as of their multiple positioning shows that in centromeres both DNA curvature and flexibility are relevant in determining nucleosomal features.

Key words: Centromere; DNA curvature; Nucleosome assembly; Yeast

1. Introduction

Centromere is an essential chromosomal element for the processes of genome replication and segregation. Common molecular features, shared among all the DNA sequences involved in centromeric functions, at the moment have not been found [1,2]. However, the sequences of centromeric DNA in budding yeasts like *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Kluyveromyces lactis* (*K. lactis*) have peculiar features shared between all the chromosomes of the same organism (16 for *S. cerevisiae* and 6 for *K. lactis*) and in relevant extent between the two yeasts [3]. In such yeasts centromeres are more compact (point centromeres) than in other biological systems and bind to only one microtubule encompassing three conserved DNA elements. Point centromeres could be a model for centromeres of high eukaryotes and fission yeasts, encompassing kilobases of DNA that are still not well clarified, at least at molecular level [1,2]. In *S. cerevisiae* and *K. lactis*, the centromeres are characterized by three DNA sequence elements called CDEI, CDEII, and CDEIII, all three essential for the centromere function. While CDEI and CDEIII, with lengths of 8–9 bp and 25–26 bp respectively, are necessary to specific protein recognition, the CDEII DNA element, characterized by an extraordinary high AT content (about 90%), has a sequence different in the two yeasts; moreover its length in *K. lactis* is twice that of *S. cerevisiae*.

The chromatin organization at centromeres is still not well clarified; so far the results obtained for *S. cerevisiae* [4,5] and for *K. lactis* [6] are consistent with the formation of a complex, about 250 bp long, resistant to nucleases such as MNase and DNAsel. These findings seem to suggest that nucleosome should not be present at the kinetochore. However a model of *S. cerevisiae* kinetochore recently proposed [1,2], hypothesizes a modified nucleosome with the histone H3 variant Cse4p assembled on CDEII. This model while supported by some genetic results [1], at the moment, lacks of direct experimental evidence.

On the basis of this model and on account of the difficulty to explore the chromatin structure at the kinetochore, due to its inaccessibility to specific nucleases, the usefulness of an in vitro approach clearly emerges. This can be achieved adopting a simplified model system, constituted by centromeric DNAs and histone octamer. This system allows to study the ability of centromeric sequences to organize nucleosomes as well as the evaluation of the stability and the structural features of centromeric nucleosomes, in comparison with bulk nucleosomes.

In this research we will show that two centromeres of *K. lactis* (KlCEN1) and *S. cerevisiae* (SCEN6) are able to organize stable nucleosomes with an high mobility in vitro, suggesting that a nucleosomal structure, at the kinetochore, although difficult to put in evidence, however is possible.

It is worth noting that KlCEN1 and SCEN6, on account of the striking similarity between all the centromeric DNA sequences in each yeast, could be considered as prototypes of *S. cerevisiae* and *K. lactis* centromeres.

2. Materials and methods

2.1. Centromeric DNAs from K. lactis and S. cerevisiae

The *K. lactis* centromeric DNA of the chromosome 1 (KlCEN1) was derived from the plasmid pKlCEN1-II.9, whose characteristics are reported in [7] (kindly provided from A.A. Winkler and B.J.M. Zonnenveld of Yeast Genetics Clusius Laboratorium at Leiden University). The centromeric DNA of *S. cerevisiae* chromosome 6 (SCEN6) was derived from the plasmid pAS2, whose characteristics are reported in [8] (kindly provided from L. Panzeri of Dipartimento di Genetica e Microbiologia at University of Milano).

KlCEN1 (218 bp) and SCEN6 (216 bp) corresponding to the cen-

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tromere fragments reported in Table 1, have been subcloned in the *Eco*RI-*Bam*HI site of pUC18 (pKlCEN1 and pSCEN6). Plasmid pXbs201, carrying the 249 bp *Xenopus borealis* somatic 5S RNA gene inserted in the *Hin*dIII-*Bam*HI site of pUC18 [9] was a gift of Ida Ruberti, Dipartimento di Genetica e Biologia Moleculare, University of Roma "La Sapienza". The DNA fragments, used for polyacrylamide gel retardation measurements, were PCR amplified from pKlCEN1 and pSCEN6 or restricted with *Eco*RI and *Bam*HI. Their sequences as that of 5S RNA gene fragment, were checked on both strands by Sanger sequencing method.

2.2. Electrophoretic mobility assay

Endlabeled DNA fragments were run on a native 8% polyacrylamide gel in 1×TBE buffer (90 mM Tris-Borate, 2 mM EDTA pH = 8.3) at a constant voltage of 4 V/cm. Gels were dried and autoradiographed and the electrophoretic mobility of the samples was then compared with that of pUC18 cleaved with *Hpa*II as molecular weight standard.

2.3. Competitive nucleosome reconstitution

The procedure used for competitive reconstitution was that of Shrader and Crothers [10,11] with minor modifications. 1.74 μg of H1 and H5 depleted nucleosomes, obtained from chicken erythrocytes, was mixed with 150 ng of radiolabeled centromeric or 5S RNA gene at various amounts (1, 3, 5 μg) of sonicated calf thymus DNA as competitor in 1 M NaCl, 10 mM Tris-HCl, 0.5 mM EDTA pH 8, 0.1% Nonidet P-40, 0.3 mM PMSF, 10 μg/ml BSA (bovine serum albumin) in a final volume of 10 μl. After incubation at room temperature for 30 min the salt concentration was lowered to 0.1 M NaCl, step by step with three additions of the same buffer without NaCl. Samples were then resolved on 5% polyacrylamide gels electrophoresis. The relative quantities of complexed and naked DNA were assayed by scanning dried gels with an Instant Imager (Packard). The free energy for KlCEN1 and SCEN6 DNA fragments were calculated from the equation $\Delta G(Centr) = RT \ln[\alpha(Nuc)] - RT \ln[\alpha(Centr)]$, where α(Nuc) is the ratio of labeled reconstituted nucleosome to labeled naked DNA for bulk nucleosome and α(Centr) is the analogous ratio for centromeric DNA fragments or for 5S RNA gene. Each fragment was reconstituted at least in three separate experiments and the results were averaged. The reproducibility of the experiments was within ~0.1 kcal/mol. The formation of a canonical nucleosome was checked by micrococcal nuclease digestion, in fact MNase trimmed the complex to a DNA fragment 146 bp long as assayed by 5% gel electrophoresis (not shown).

3. Results and discussion

3.1. Theoretical and experimental evaluation of centromeric DNA superstructural features show that, in spite of their very high AT content, KlCEN1 and SCEN6 are not highly curved

The superstructural features of the two DNA fragments KlCEN1 and SCEN6 were derived adopting a theoretical method, previously developed by us [12], based on the integration of the slight deviations from the standard B conformation of the 16 possible dinucleotides, calculated by energy minimization. The result reported in Fig. 1A as stereoviews, shows that the KlCEN1 presents a unique in phase curvature, while SCEN6 presents a sequence of three slightly curved tracts out of phase. In both cases the extraordinary AT richness of the CDEII region of the centromeres does not give rise to a large DNA curvature as that found, for example, in *Crithidia fasciculata* [13]. It is interesting to note that the two DNAs appear to be characterized by locally high curved DNA tracts with different phase. This feature is more relevant in the case of SCEN6 as it is possible to observe in the curvature diagrams where the curvature vector is reported as modulus and phase along the nucleotide sequence (not shown). The superstructural features of KlCEN1 and SCEN6, theoretically predicted, resulted in good agreement with experimental evaluation, obtained by polyacrylamide gel electrophoresis. In fact as reported in Fig. 1B, both DNA centromeric fragments appear retarded with respect to their real molecular weights. Their retardation ratios are respectively R^{EXP}KlCEN1 = 1.19 and R^{EXP}SCEN6 = 1.06. The theoretical retardation ratios, calculated as previously described, are respectively R^{TH}KlCEN1 = 1.18 and R^{TH}SCEN6 = 1.07. These values are increased in case that the gel retardation was measured in the presence of the aliphatic polyamine spermine, a molecule that was previously shown to be able to increase DNA curvature [14], and are respectively R = 1.21 and R = 1.11, suggesting that in vivo,

Table 1
Comparative free energies in nucleosome formation relative to average sequence nucleosomal DNA

DNA	Sequence	ΔΔG (Kcal/mole of nucleosome)
	---CDEI----------CDEII----------CDEIII---	
	⇓ ⇓ ⇓	
SCEN6	* F--ATCACGTG-[86bp 95% AT] -AGTTTTTGTTTTCCGAAGATGTAAA--F'	- 1.50
	⇓ ⇓ ⇓	
KlCEN1	--TGCACGTGA-[161bp 87% AT]-TGCTTTATGTTTCCGAAAATTATTTT--	- 2.00
5S RNA gene from *Xenopus borealis*		- 1.65
Mononucleosomal DNA		0

*F and F′ indicate flanking sequences of *S. cerevisiae* centromere 6 to obtain a fragment length equal to that of *K. lactis* centromere 1.

the centromere curvature could be more relevant with respect to the values, measured in vitro, taking into account to the presence of Mg^{2+} cations as well as polyamines in the cells.

3.2. *Competitive nucleosome reconstitution of KlCEN1 and SCEN6 shows that both sequences are able to organize a stable nucleosome, with multiple translational positions*

To evaluate the propensity of KlCEN1 and SCEN6 in forming nucleosomes, a competition for a limited number of histone is established between radiolabeled centromeric DNA and unlabeled, heterogenous sequence DNA. The fraction of labeled DNA present in the nucleosomal band depends on the intrinsic ability of that sequence to reconstitute in nucleosome compared to bulk DNA. Naked DNA and nucleosomal complex were separated on a 5% polyacrylamide gel, as shown in Fig. 2A. The relative fractions of labeled DNA in the nucleosomal and naked DNA bands were used to calculate the free

Fig. 1. A: Stereoprojections of KICEN1 superstructure (on the top) and of SCEN6 superstructure (on the bottom). B: 8% polyacrylamide gel electrophoresis of KlCEN1 (lanes 1, 4) and SCEN6 (lanes 2, 5). M, pUC18 digested with *Hpa*II (lane 3). The samples were internally labeled by PCR (lane 1 and 2) or endlabeled by polynucleotide kinase (lanes 4 and 5).

energy difference of nucleosome formation on centromeric sequences relative to average sequence DNA.

For sake of comparison, we have studied in the same experimental conditions the *X. borealis* 5S RNA gene, a sequence well known for forming a very stable nucleosome. In this case, we have found a ΔG value in fairly good agreement with that previously reported by Wolffe and coworkers [15], see Table 1. Both KlCEN1 and SCEN6 are able to organize a very stable nucleosome, the first centromere even more stable than that formed by 5S RNA gene, while the SCEN6 nucleosome has a ΔG value slightly lower than 5S RNA gene.

An interesting feature is the presence of a number of bands for reconstituted nucleosome shown by all the three examined sequences on 5% polyacrylamide gel electrophoresis (see Fig. 2A). This feature of the nucleosome band shift is more easily detectable by the densitometric analysis of the complex bands, as shown in Fig. 2B.

In all cases five bands with different intensities are present. We attribute the band 4 to the nucleosome more populated position, while the band 5 is probably due to a complex of nucleosome with octamer, since it decreases and then disappears increasing the competitor DNA. Bands 1, 2, 3 should correspond to other nucleosome translational positions differently populated. While further analyses with different methods are necessary to precisely map the different nucleosome positions along the DNA sequences, however the precise correspondence between the electrophoretic mobilities of the bands behaving to 5S RNA gene and to KlCEN1 and SCEN6 strongly suggests that the three sequences are all able to organize multiple nucleosome positioning with the same rotational phase, as previously shown in case of 5S RNA gene [16].

3.3. *The comparison between experimental and theoretical nucleosome organization on KlCEN1 and SCEN6 reveals that both DNA curvature and DNA flexibility are relevant in determining nucleosome formation free energy*

By extending the model successfully advanced in predicting the sequence dependent circularization propensity of DNA tracts [17], an analytical formulation was obtained for evaluating the nucleosome positioning along the DNA tracts investigated, as well as the free energy difference between the

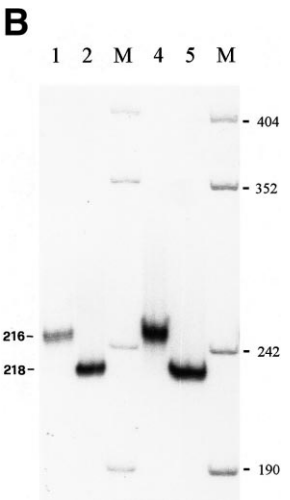

Fig. 1. (*Continued*)

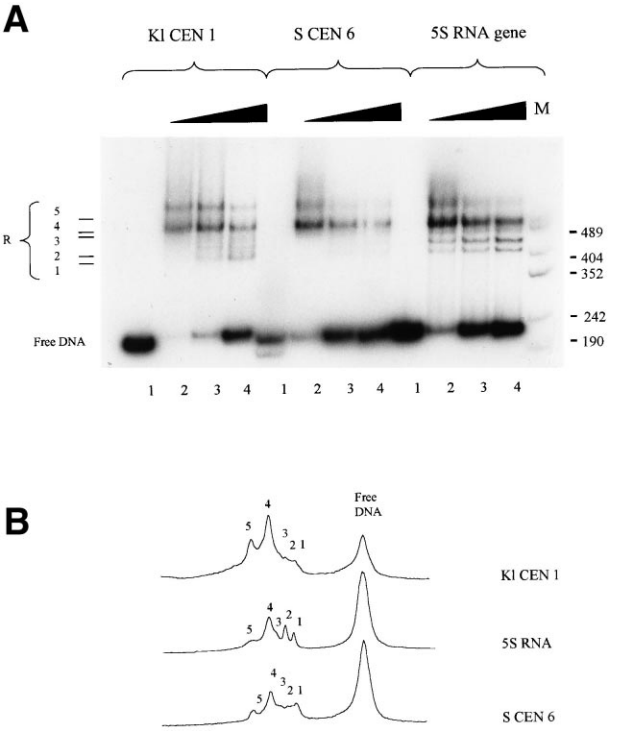

Fig. 2. A: 5% polyacrylamide gel electrophoresis of nucleosomes reconstituted on KlCEN1, SCEN6 and 5S RNA gene in the presence respectively of 1, 3 and 5 μg of competitor DNA (lanes 2, 3, 4). Naked DNA (lane 1). R stands for nucleosome reconstituted DNA. B: Densitometric profiles of lanes 4.

studied sequence and a reference sequence in nucleosome competitive reconstitution (De Santis et al., unpublished). This corresponds to locate the minimum of the free distortion energy of recurrent 145 bp DNA tracts from their curvature. The calculations involve both the sequence dependent curvature and flexibility. DNA flexibility has been derived on the basis of the melting temperatures of the DNA tracts examined, calculated from their base composition [18].

The results are shown in Fig. 3, where the elastic free energy of nucleosome formation is reported versus the DNA nucleotide sequence and allows to localize the nucleosome dyad axis positions on the two centromeres; for comparison also the nucleosome free energy of 5S RNA gene is reported. It is interesting to note that all the three DNA tracts are able to organize nucleosomes with multiple translational positions with the same rotational phasing. Namely different translational positions of rotationally phased dyad axis characterize the nucleosome on KlCEN1 which is clearly the centromere able to organize the most stable nucleosome; 5S RNA gene nucleosome positions correspond substantially to those of KlCEN1 except that the nucleosome free energy is higher. In the case of SCEN6 the number of nucleosome positions in phase is lower and their energy is similar to that of 5S RNA gene and higher than that of the other investigated centromere. The theoretical differences between nucleosome free energies, for the three sequences, are in good agreement with the experimental measurements in Table 1. Furthermore the presence of multiple nucleosome translational positions, theoretically predicted, in Fig. 3, is in agreement with experimental measurements. In fact, seven nucleosome positions, as theoretically predicted, if equally spaced along 220 bp should

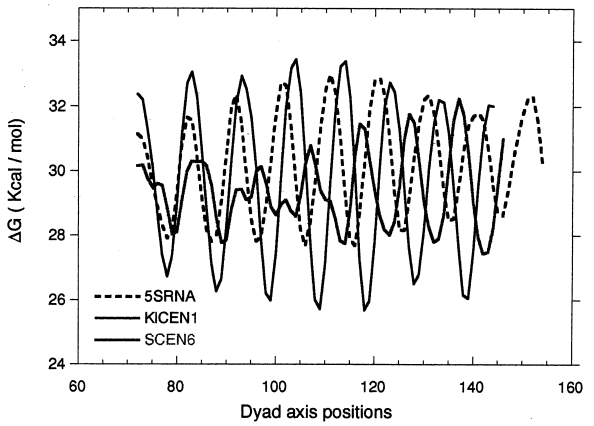

Fig. 3. Elastic distortion free energy of recurrent 145 bp DNA tracts versus the dyad axis positions along the sequence number. The free energy values are calculated in kcal/mol of nucleosome.

give rise to 3+1 differently migrating electrophoretic bands, due to 3×2 nucleosome positions equidistant from the DNA fragment ends, plus 1 central position as experimentally found (see Fig. 2A and B). The main conclusion that can be derived from the reported results is that DNA flexibility is relevant as well as DNA curvature in determining nucleosome stability. In fact two typical centromeric DNAs like KlCEN1 and SCEN6, that are not highly curved in spite of their very high AT content, are able to organize nucleosome as stable as that formed on 5S RNA gene, so far considered as one of the more stable nucleosomes. This derives from the high flexibility of the two centromeric sequences, as deduced from an original theoretical method recently developed in our laboratory, which calculated nucleosome free energy of formation taking into account both DNA curvature and flexibility. The fairly good agreement between theoretical and experimental results is a significative support to the relevance of both DNA features on nucleosome organization.

Finally, the presence of nucleosome at the kinetochore, although to be assayed in vivo, however, is well possible, as suggested by the values of the nucleosome free energies, reported in the present paper.

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References

[1] Bloom, K., Hill, A., Kenna, M. and Saunders, M. (1989) Trends Biochem. Sci. 14, 223–227.
[2] Pluta, A.F., Mac Kay, A.M., Ainsztein, A.M., Goldberg, I.G. and Earnshaw, W.C. (1995) Science 270, 1591–1594.
[3] Heus, J.J., Zonneveld, B.J.M., Steensma, H.Y. and van den Berg, J.A. (1993) Mol. Gen. Genet. 236, 355–362.
[4] Bloom, K.S. and Carbon, J. (1982) Cell 29, 305–317.
[5] Funk, M., Hegemann, J.H. and Philippsen, P. (1989) Mol. Gen. Genet. 219, 153–160.
[6] Heus, J.J., Bloom, K.S., Zonneveld, B.J.M., Steensma, H. and Van den Berg, J.A. (1993) Chromosoma 102, 660–667.
[7] Heus, J., Zonneveld, B.J.M., Steensma, H.J. and Van den Berg, J.A. (1990) Current Genet. 18, 517–522.
[8] Panzeri, L., Laudonio, L., Stotz, A. and Philippsen, P. (1985) EMBO J. 4, 1867–1874.
[9] Razvi, F., Gargiulo, G. and Worcel, A. (1983) Gene 23, 175–183.
[10] Shrader, T.E. and Crothers, D.M. (1989) Proc. Natl. Acad. Sci. USA 80, 7418–7422.
[11] Shrader, T.E. and Crothers, D.M. (1990) J. Mol. Biol. 216, 69–84.
[12] Cacchione, S., De Santis, P., Foti, D., Palleschi, A. and Savino, M. (1989) Biochemistry 28, 8706–8713.
[13] Kropp, B., Leoni, L., Sampaolese, B. and Savino, M. (1995) FEBS Lett. 364, 17–22.
[14] Bordin, F., Cacchione, S., Savino, M. and Tufillaro, A. (1992) Biophys. Chem. 44, 99–112.
[15] Godde, J. and Wolffe, A.P. (1996) J. Biol. Chem. 271, 15222–15229.
[16] Pennings, S., Meersseman, G. and Bradbury, E.M. (1991) J. Mol. Biol. 220, 101–110.
[17] De Santis, P., Fuà, M., Savino, M., Anselmi, C. and Bocchinfuso, G. (1996) J. Phys. Chem. 100, 9968–9976.
[18] Gotoh, O. and Takashira, Y. (1981) Biopolymers 20, 1033–1042.