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Geometrical, conformational and topological restraints in regular nucleosome compaction in chromatin

Anita Scipioni, Giulia Turchetti, Stefano Morosetti, Pasquale De Santis *

Dipartimento di Chimica, Sapienza Università di Roma, P.le A. Moro, 5 I-00185 Roma, Italy

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ABSTRACT

The folding of the nucleosome array into a chromatin fiber modulates DNA accessibility and is therefore an important factor for the control of gene expression. The statistical analysis of the nucleosome repeat length in chromatin fibers reveals the presence of a ten-fold periodicity suggesting the existence of orientational constraints of the nucleosome units that provide the geometrical conditions of helical conformations. Recently, the elucidation of the x-ray crystal structure of a nucleosome tetramer array and the interpretation of electron microscopy images of reconstituted nucleosome arrays suggested two different architectures of the chromatin fiber. We approached the problem by integrating the experimental findings with geometrical, conformational and topological restraints, under the hypothesis of the minimum distortion of the nucleosome and linker DNA structures. We show that the excluded volume at linker crossing and the torsional energy limit the possible close packing of the nucleosomes in the chromatin fiber. In particular, the torsional energy of the chromatin fiber appears crucial in determining the kind of nucleosome packing for short nucleosome repeat lengths as in telomeres and yeast chromatin.

1. Introduction

Chromatin architecture is the substrate for DNA replication, recombination, transcription and repair and plays an important role in the regulation of nuclear processes of genomes. It is the result of complex hierarchic assemblages of nucleosome arrays in a compact structure. Although the structure of the chromatin fundamental unit, the nucleosome, is known in its molecular details at near-atomic resolution [1], the basic information about the pattern of the organization of nucleosomes in the chromatin fiber is still debated.

Compaction of nucleosomes in chromatin is directed by their positioning along DNA and therefore by the length of the DNA linkers that bridge and mutually orient the adjacent nucleosomes in the space. Their average length varies among species, cell types within the species as well as among nucleosomes within the same cell type. However, a statistical analysis of their length distribution in chromatin reveals that the nucleosome repeat length (NRL) differs in multiples of about 10 bp, close to the DNA helical repeat [2]. Such a finding suggests the existence of orientation constraints between the nucleosomes and between the nucleosomes and the fiber axes.

Recently, two different models of the chromatin fiber were proposed [3,4]. The former was obtained solving the x-ray crystal structure of a tetranucleosome at a 9 Å resolution [3] while the latter was the result of interpretations of EM and cryo-EM images of reconstituted nucleosome arrays containing linker histone [4]. Both the chromatin fiber models were obtained using DNA periodical constructs resulting from multimeric tandem ligations of the strongly nucleosome positioning 601 Widom sequence [5]. In the first case the length of the DNA repeat unit was 167 bp [3], whereas several quantized linker lengths were investigated in the EM experiments corresponding to the NRLs from 177 to 237 bp with a fixed increment of 10 bp [4].

The crystal structure of the tetranucleosome shows that linker DNA zigzags back and forth between two stacks of nucleosome cores. Adopting the tetranucleosome x-ray structure as a repeating module of the 30-nm chromatin fiber, double super-helix architecture can be obtained in agreement with the two-start helix model suggested by the disulfide cross-linking experiments [6].

This nucleosome organization, however, is not consistent with the EM experiments that are characterized by a higher nucleosome packing ratio, ranging between 11 and 17 nucleosomes per 11 nm, and interpreted with an interdigitated model [4]. Furthermore, as pointed out by Robinson et al. [4], the Richmond model [3] does not explain the apparent transition of the nucleosome density along the fiber and its radial dimension occurring at multimeric DNA periodicity between 207 and 217 bp consequent to the modular increasing of linker length by 10 bp.

Such a debated question about the chromatin fiber structure was very recently made more complex by Ghirlando and Felsenfeld [7] and Grigoryev et al. findings [8]. Hydrodynamic studies on defined natural heterochromatin fragments of chicken erythrocytes support a chromatin fiber having about 6–7 nucleosomes per 11 nm [7]. Grigoryev et al. show

* Corresponding author. Tel./fax: +39 06 4453827.
E-mail addresses: anita.scipioni@uniroma1.it (A. Scipioni), giulia.turchetti@uniroma1.it (G. Turchetti), stefano.morosetti@uniroma1.it (S. Morosetti), pasquale.desantis@uniroma1.it (P. De Santis).

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that linker histones lead to a two-start zigzag dominated by interactions between alternate nucleosomes with a maximum packing ratio of 8 nucleosomes/11 nm for nucleosome arrays of 12 residues with 207 bp repeats [8]. These results suggest much less compact chromatin structures than those resulting from EM experiments on reconstituted chromatin models [4].

Very recently, Routh et al. [9] showed that the compaction is both NRL- and linker histone-dependent comparing the compaction of long reconstituted nucleosome arrays with different NRLs (167 and 197 bp). Binding of H5 gives rise to a highly regular and compact fiber with a diameter of 33–35 nm for the197-bp NRL array, whereas a less compact fiber with a diameter of 21 nm is obtained for the 167-bp NRL array. Importantly for understanding the regulation of compaction, the shorter NRL array shows a two-start helix arrangement characterized by stacking of nucleosome cores similar to that found in the tetramer x-ray structure [3].

In spite of these relevant experimental results [3,4,6–9], the possible architectures of the native chromatin fiber remain an unresolved question. The path of the DNA linkers and then the sequential organization of nucleosomes are a relevant missing piece in our understanding of the chromatin fiber folding. Different theoretical and computational approaches were proposed to this aim [10–18].

Monte Carlo simulations were carried out to investigate the different conformations that regular nucleosome arrays assume under the restraints of nucleosome core attractions and conformational entropy of the chromatin chain [10–12]. Adopting ellipsoid potential, first introduced by Wedeman and Langowski [10], and spherocylinder Zewdie potential, Stehr et al. [11] and Kepper et al. [12] investigated the effect of different internucleosomal interaction strengths on the fiber conformations. Although this approach gave rise to a large number of slightly compact structures, the simulations revealed the importance of stacking, geometry and repeat length of nucleosomes on the compaction of the chromatin fiber.

Daban and Bermúdez [13] proposed an interdigitated solenoid model for compact chromatin fibers to explain previous EM visualizations of small chromatin fragments from chicken erythrocytes [14].

Wong et al. [15] reported a model of chromatin fiber evaluating the different arrangements of idealized linkers capable of joining the entry–exit nucleosomal DNA and compacting them in cylindrical surfaces as rectangular boxes.

Staynov and Proykova [16,17] analyzed different helical models selecting two regular non-sequential arrangements of negatively tilted nucleosomes along the fiber helix in account of the DNase digestion pattern of chicken erythrocyte chromatin.

Depken and Schiessel [18] recently addressed the question by constructing a simple geometrical model based on the nucleosome wedge shape constrained to cover a cylindrical puzzle to predict the EM dimensions of the condensed chromatin fiber.

The aim of this paper is to identify possible molecular models of compact chromatin fibers by integrating the experimental data adopted as constraints (nucleosome density and helical diameter) with geometrical, conformational and topological restraints.

The thermodynamic stability of the chromatin fiber, constituted by nucleosome large and compact units connected by the relatively thin arms of the linkers, implies close packing of the nucleosome cores, which should sustain the large part of Brownian perturbations on the nucleosomes. We propose an approach based on the assumption that the short-range attraction between the nucleosome cores constitutes the dominant interaction in compact chromatin fibers, as proposed very recently by Depken and Schiessel [18]. However, despite of possessing the intrinsic ability to condense through the action of the core histone interactions, folded states of nucleosomal arrays are found relatively unstable in the absence of linker histones, which selectively neutralize and constrain the entering and exiting linker DNA [8,19]. Carruthers et al. [19] showed that linker histones stabilize chromatin folding but do not appear to directly contribute to the internucleosomal interactions required for chromatin folding. Moreover, single-molecule force spectroscopy measurements revealed that linker histones do not affect the length of the fiber but stabilize its folding [20].

The conformational analysis reported here investigates the compaction of regular arrays of nucleosomes reconstituted on multimeric DNA constructs, assuming the principle of conformational equivalence, and identifies the possible helical structures. Such structures are models of natural chromatin tracts that show evidence of quasi-regular fibers, implicitly considering those stabilized by linker histones.

2. Materials and methods

2.1. Geometrical considerations

The periodical repeats of nucleosomes can be investigated assuming the principle of conformational equivalence of the repeating units. The problem of selecting the compact architectures of the chromatin fiber compatible with a given orientation of nucleosomal axes with respect to the fiber axis for different linker lengths can be factorized in one in which only orientational parameters are taken into account and the other in which the lengths of DNA linkers are considered. The latter step does not change the fiber periodicity and the mutual orientation of nucleosomes. However, it can change the fiber metrics, namely the inter-nucleosome distances, under the realistic hypothesis of the invariance of the nucleosome structure. Therefore, assuming the conformational equivalence of the repeating units, compaction of nucleosomes in chromatin should be strongly restricted by geometrical constraints, which eventually determine the fiber architecture due to the large overcrowding of the structure.

As generally adopted for the polymers, the helical structures are conveniently classified by the ratio n/t where n is the number of nucleosomes and t is the number of turns in the repeating structural period (t is positive for a right-handed helix). The helical parameters, namely, the cylindrical repeat angle $\phi = 2\pi t/n$ and the monomer repeat on the helical axis, as well as the external helical diameter are analytically related to the entry–exit angle, the torsional angle around the linker virtual axis, and the linker length, adopting the matrix formulations early introduced in the conformational analysis of synthetic polymers, polypeptides and polynucleotides [21]. Adopting a Cartesian reference on a nucleosome unit and another one equivalent on the next along the polynucleosome chain, the coordinates of this unit in its Cartesian reference are related to those of the preceding unit by a rotation matrix obtained as the product of the pure rotation matrices of torsional angles around the chain bonds and bond angles following the DNA chain connecting the two nucleosome units. A rotation matrix in terms of the DNA curvature and phase angles, which determine the mutual orientation of successive base pairs along the DNA, can be adopted as an appropriate approximation [22].

The matrix **A**, which transforms a repeating unit into the next, is defined as

$$\mathbf{A} = \begin{vmatrix} a_{11} & a_{12} & a_{13} \\ a_{21} & a_{22} & a_{23} \\ a_{31} & a_{32} & a_{33} \end{vmatrix} \tag{1}$$

By an appropriate similarity transformation, the final rotation matrix **A** will be in the diagonal form with eigen-values: $e^{i\phi}$, $e^{-i\phi}$, 1, whose trace is $2\cos\phi + 1$, where ϕ is the cylindrical angle around the helical axis. It corresponds to the eigen-vector of the eigen-value 1, whose coordinates are easily obtained from the matrix equation

$\mathbf{Ah} = \mathbf{1\,h}$, where $\mathrm{h} = \frac{1}{N}\begin{vmatrix} a_{32}-a_{23} \\ a_{13}-a_{31} \\ a_{21}-a_{12} \end{vmatrix}$ and N is the normalization factor.

This means that the helical axis is the same for all the coordinate systems associated to the nucleosome units. Since the principle of

conformational equivalence is assumed, the nucleosome dyad symmetry requires that the dyad axis, **d**, is perpendicular to the helical axis. In fact, if the *x*-axis of the Cartesian system is fixed on the dyad axis of a nucleosome unit, it is related to those fixed on the adjacent units by both the helical axis and the dyad.

If the vector **d** is $\begin{vmatrix}1\\0\\0\end{vmatrix}$, then $\mathbf{Ad} = \begin{vmatrix}a_{11}\\a_{21}\\a_{31}\end{vmatrix}$ and

$$\mathbf{D} = \begin{vmatrix}1 & 0 & 0\\0 & -1 & 0\\0 & 0 & -1\end{vmatrix} = \tilde{\mathbf{D}} \tag{2}$$

where **D** is the rotation matrix around the dyad axis. Due to the properties of the matrices **A** and **D**, **ADAD** = **I** (where **I** is the identity) and

$$\mathbf{A} = \mathbf{D}\tilde{\mathbf{A}}\mathbf{D} = \begin{vmatrix}a_{11} & -a_{21} & -a_{31}\\-a_{12} & a_{22} & a_{32}\\-a_{13} & a_{23} & a_{33}\end{vmatrix} \tag{3}$$

where $a_{12} = -a_{21}$, $a_{13} = -a_{31}$, and $a_{23} = a_{32}$. As a consequence,

$$\mathbf{h} = \frac{1}{N}\begin{vmatrix}0\\2a_{31}\\-2a_{21}\end{vmatrix} \tag{4}$$

and the scalar products $\mathbf{h}\cdot\mathbf{d}$, $\mathbf{h}\cdot\mathbf{Ad}$, $\cdots\cdots$ $\mathbf{h}\cdot\mathbf{A}^n\mathbf{d}$ vanish.

Therefore, the presence of a dyad axis, which relates the two halves of the nucleosome repeat unit along the chromatin fiber, imposes that the dyad is perpendicular to the helical fiber axis as a result of the conformational equivalence of the repeating units. As a consequence, the repeating unit is halved, the possible helical structures are uniform and the twist angles around the linker axes of the nucleosome DNA entry and exit should be equal.

This could be not strictly true for a real chromatin fiber. However, since the nucleosome packing requires the quasi-orientational order (quasi-helical structure), the linkers should differ by a multiple of 10 bp only, and the dyad axes would result almost perpendicular to the fiber axis. In this respect, the analysis of the x-ray crystal structure of the nucleosome tetramer [3] reveals the existence of a quasi-helical axis, perpendicular to the crystallographic dyad axis, which reports nucleosome pairs, and quasi-perpendicular to the nucleosome dyads, in spite of the crystal packing perturbations and the end effects.

Therefore, the experimental evidence that the nucleosome dyad axis in natural chromatin is nearly perpendicular to the fiber axis [23] is a result of the "quasi-conformational equivalence" of the repeating units and of the two halves of the nucleosome, even if linker lengths are not strictly equal. Therefore, the linkers connecting the adjacent nucleosome cores could be differently arranged but within the structural and topological boundaries allowed by the packing of the nucleosomes.

The extensive inter-nucleosome interactions, which characterize the nucleosome compaction in the chromatin fiber, could explain the observed structural transition occurring in the axial and radial nucleosome density of regular nucleosome assemblies by increasing their spacing along DNA by steps of 10 bp [4]. In fact, the 10 bp increment is slightly different from the 10.5 bp helical repeat of DNA in solution; it implies a torsional stress that could be followed by a transition to a different packing arrangement when a critical length is reached. On the other hand, the path of the linkers in compact nucleosome packing is also severely constrained by steric hindrances and topological conditions.

Fig. 1 schematically illustrates the adopted geometrical features of a helical fiber structure. The green and red dots indicate the entry (down) and exit (up) DNA axis of the nucleosome core. The linkers are modeled as conformationally equivalent straight segments corresponding to "virtual bonds" connecting consecutive nucleosome units reported by screw symmetry along the fiber axis. These "virtual linkers" determine the mutual position and orientation of the nucleosome cores. The helical structures produced are almost equivalent for the nucleosome core packing and integral twist of real linkers.

The virtual linker length determines the fiber diameter for a given helical class and the nucleosome axial density in terms of the nucleosome tilt angle, η, around the nucleosome dyad axis (see Fig. 1A). The vector **s** connects the entry–exit DNA axes of the nucleosome core; the situation when **s** is parallel to the fiber axis is adopted as $\eta = 0$. In this representation of the virtual helical chain, the right-handed and left-handed conformations are symmetry related by a mirror, which changes the sign of the rotation angles. Apparently, this symmetry does not work for the nucleosome core (see Fig. 1B); the right and left-handed chromatin fibers having the same diameter and helix pitch are no longer equivalent for what concerns the packing of the nucleosome cores in spite of the equivalence of the mutual arrangements of the linkers. It should be noted that the intrinsic chirality of the nucleosome appears to be more suitable for right-handed helices of the linker chain connecting the up position of a nucleosome to the down of the next, since a smaller curvature distortion of the linkers is required (see Fig. 1B and C). On the contrary, the opposite pitch of the linkers is more suitable for left-handed helices. Due to the dyad symmetry of the nucleosome, the rotation of 180° around its dyad axis does not modify the orientation and packing of the nucleosomes. However, the entry and exit linkers cross following the interchange of the down and up positions, as illustrated in Fig. 1C.

2.2. Linker arrangements in compact chromatin fibers

The possible arrangements of the linkers within the framework of the helical classes with fixed radial and axial nucleosome density were analyzed in terms of the nucleosome tilt angle, η. Since $\eta = 0°$ corresponds to the vector **s** parallel to the fiber axis, $\eta = -25°$ represents the nucleosomal DNA super-helical axis perpendicular to the fiber axis (see Fig. 1B).

Due to the axial nucleosome density, setting up the fiber diameter determines the dimensions of the internal cylinder where the linkers are located (the diameter equal to the fiber diameter minus 22 nm and height equal to 11 nm). Geometrical calculations permit obtaining the distances between the linker axes at the crossing points in the axial projection in terms of the tilt angle η for all the helical classes with n ranging within 6 and 17 according to the experimental nucleosome density values [3,4,7,8]. Fig. 2A shows the scheme of the entry (l_{en}) and exit (l_{ex}) virtual linkers of vicinal nucleosomes. The mth nucleosome represents that adjacent to the assumed starting nucleosome (0-th) in the fiber. It can be located at the right (m positive) or at the left (m negative) of the starting nucleosome in the direction of the rising helix. m is related to the number of nucleosomes, n, and turns, t, according to the equation $m = \mathrm{int}(i\cdot n + 1)/t)$, where i is a positive or negative integral number suitably chosen to have the smallest integral value of m. Due to the rotation around the nucleosome dyad axis, a third crossing involving the virtual linkers $l_{en}(0)$ and $l_{ex}(0)$ has to be considered for values of the tilt angle between 70 and 120°.

Fig. 2B illustrates the crossing distance diagram of the linkers in the case of 11/4 and 17/7 helix classes, as examples, adopting the values of 34 and 44 nm for the fiber diameter, respectively, according to the electron microscopy findings [4]. An entry–exit distance, s, of 7 nm was adopted as the distance between the linker DNA arms when they leave the histone core surface as resulting from the nucleosome x-ray crystal structure [1]. To test the sensitivity of this parameter, a significantly reduced distance of 5 nm was used to simulate a more folded DNA around the histone core. However, the comparative analysis of the crossing distance profiles does not show significant changes.

The need to avoid steric hindrances implies distances greater than 2.5 nm between the linker helical axes at the crossing in the axial projections. This value corresponds to the average distance of B-DNA axes

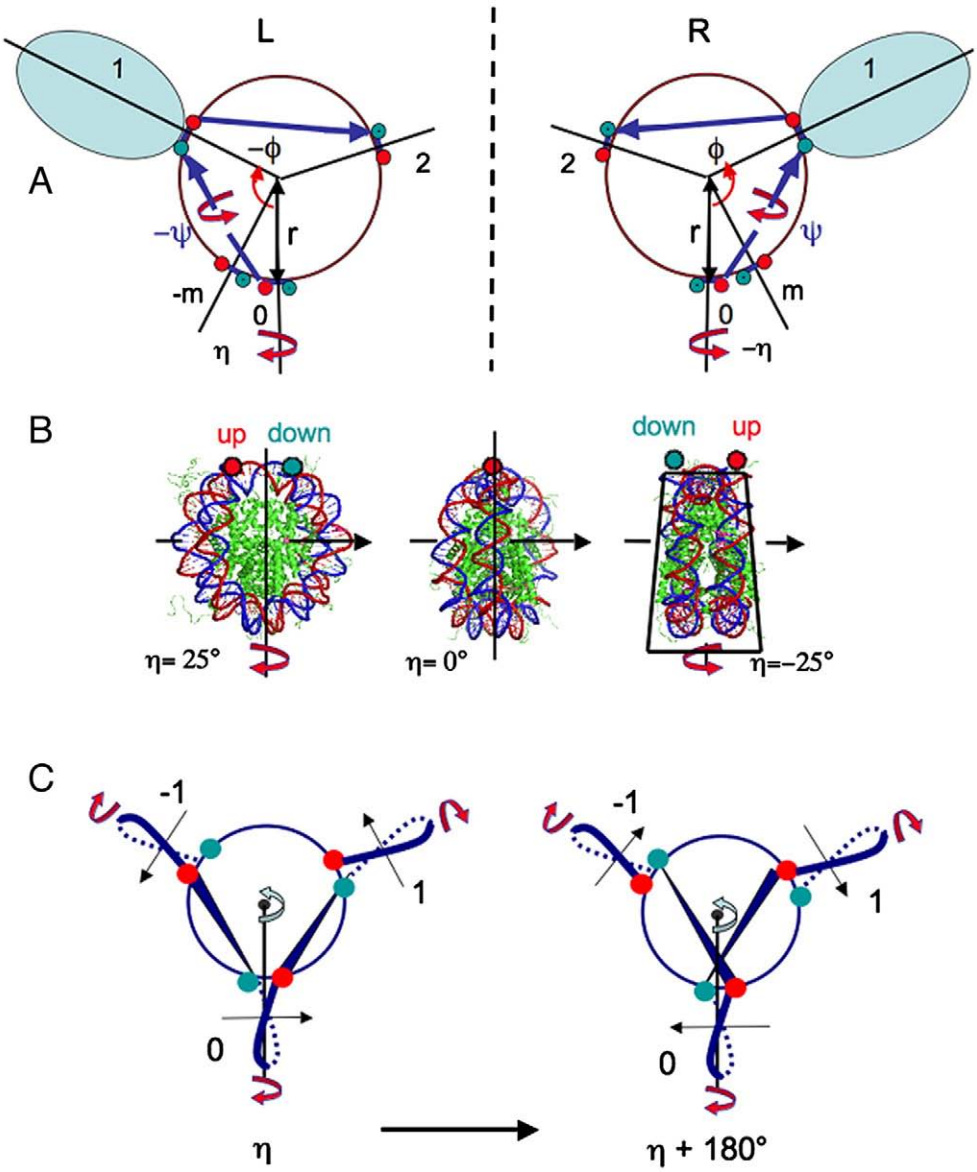

Fig. 1. Geometrical features of a helix fiber. (A) The green and red dots indicate the entry (down) and exit (up) DNA axis of the nucleosome core. The linkers are represented as straight segments corresponding to virtual bonds connecting the successive nucleosome units reported by screw symmetry along the fiber axis. η is the rotation angle around the nucleosome dyad axis (tilt). The right-handed and left-handed conformations are symmetry related by a mirror; (B) **s** is the vector connecting the entry–exit DNA axes of the nucleosome core; **s** parallel to the fiber axis is adopted as $\eta = 0°$ (center of the figure). The rotation of the nucleosome of $\eta = 25°$ and $-25°$ shows that the mirror symmetry does not hold for the packing of the nucleosome core. In particular, when $\eta = -25°$, the nucleosomal DNA super-helical axis is perpendicular to the fiber axis; (C) the entry and exit linkers cross following the interchange of the down and up positions after a rotation of 180° around the nucleosome dyad axis. Due to the dyad symmetry, the rotation does not modify orientation and packing of the nucleosomes.

as found in the x-ray crystal structures of a large number of oligonucleotides [24]. In addition, topological conditions require that the vectorial distances have the same sign since a linker cannot be located between the entry and exit linkers of other nucleosomes. This situation would produce forbidden entanglements of the nucleosome units in the fiber.

The crossing distance profiles in Fig. 2B refer to right-handed helices; the same diagrams hold for the corresponding left-handed helices except for the opposite sign of the angle η (see Fig. 1A). For the helix class 11/4, the diagram indicates that only two right-handed structures are allowed: one with the nucleosome axis almost perpendicular to the fiber axis at η about 150°, the other with η around −70° where the nucleosome helical axis is about parallel to the fiber axis (the blue arrows indicate the permitted structures). A wider range of η is permitted for the helix class 17/7: −180–90° and 120–180°, evidenced by the red double arrows. These angles change the signs in the case of the corresponding left-handed structures. The corresponding helix structures, obtained for a 180° rotation of the nucleosome tilt angle, are different for the linker crossings but leave unchanged the nucleosome packing due to the nucleosome dyad symmetry (see Fig. 1C).

The same analysis was performed for all the helical structures. The possible linker arrangements are very limited in all the cases, particularly for the two-start helices that are characterized by $t = 1/2 \cdot (n\text{-}1)$ (n is odd, t is positive for right-handed helices). In fact, double alternating linkers crossing near the fiber axis with a distance of $2 \cdot 11/n$, which should be greater than 2.5 nm, the closest distance between B-DNA axes [24], impose that the axial nucleosome density must be less than 8.5 nucleosomes per 11 nm to avoid the clashing of the linkers. This restraint agrees with the experimental results by Grigoryev et al. [8] that found a maximum compaction of about 8 nucleosomes/11 nm for oligonucleosomes dominated by two-start zigzag interactions between alternate nucleosomes.

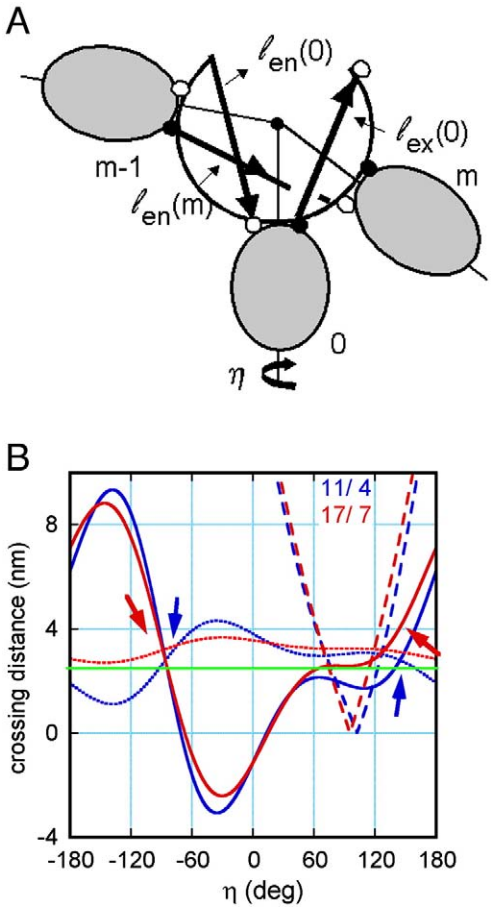

Fig. 2. Scheme of virtual linkers of vicinal nucleosomes and crossing distances profiles. (A) Entry (l_{en}) and exit (l_{ex}) virtual linkers of vicinal nucleosomes. The *m*th nucleosome is the adjacent to the starting nucleosome (0-th) in the fiber; if the *m*th nucleosome is located at right, *m* is positive, negative if at left of the starting nucleosome in the direction of the rising helix; (B) crossing distance profiles of the linkers in the case of 11/4 (blue lines) and 17/7 (red lines) helix classes as a function of the tilt angle η, adopting the values of 34 and 44 nm for the fiber diameter, respectively, and an entry–exit distance, *s*, of 7 nm. The full lines refer to the crossing distances between $l_{en}(0)$ and $l_{en}(m)$; the dot lines refer to the crossing distances between $l_{ex}(0)$ and $l_{en}(m)$; the dashed lines are for the crossing distances between $l_{ex}(0)$ and $l_{en}(0)$. The arrows indicate the permitted structures.

2.3. Conformational parameters: twist deformation of linker DNA in compact fiber structures

The rotation angle around the linker virtual axis, ψ, characterizes the helical distribution of linkers as well as that of the nucleosomes in the chromatin fibers and directly contributes to the conformational energy. This introduces a further constraint in account of the torsional energy required to obtain the different helical structures from those conformationally relaxed. It is a function of the nucleosome tilt angle, η, for each helical class and can be easily calculated adopting the cylindrical coordinate system of the fiber for any helical class n/t as:

$$\psi = \cos^{-1}\left[\frac{\mathbf{s}\times\mathbf{l}\cdot\mathbf{l}\times\Phi\mathbf{s}}{l^2 s^2 \sin\cos^{-1}(\mathbf{s}\cdot\mathbf{l}/sl)\sin\cos^{-1}(\mathbf{l}\cdot\Phi\mathbf{s}/sl)}\right] \quad (5)$$

where **s** is the vector that bridges the entry to the exit DNA axis of the nucleosome core, and **l** is the linker axis vector; Φ is the rotation matrix that transforms each nucleosome unit into the next one according to the value of the cylindrical angle ϕ around the fiber axis. The rotation angle ψ is defined as the dihedral angle between the two planes containing the entry–exit distance vectors (**s, Φs**) of the adjacent nucleosome units and the virtual linker bridging them (**l**). When the vectors **s** and **Φs** are mutually parallel and parallel to the fiber axis, the rotation angle ψ corresponds to 180°. It should be noted that the conformational angle, ψ, calculated for the virtual linkers can be associated to the actual linkers since the contribution of the writhing due to the linker curvature is low and the topological invariance between the virtual and actual fiber structure can be assumed.

Fig. 3A illustrates the trend of the conformational angle ψ in terms of the nucleosome tilt angle η for the 11/4 and 17/7 helical classes, which shows that ψ is very similar for the two helices. Analogous diagrams were obtained for all the helical classes considered.

The general feature of such diagrams is the practical linear relation between ψ and 2η. This relation becomes progressively more valid when the cylindrical angle ϕ around the fiber axis increases approaching 180°. The different helical classes can be further characterized by the orientation of nucleosome super-helical axes with respect to the fiber axis as: trans-like (the nucleosome axes are perpendicular to the fiber axis), cis-like (the nucleosome axes are parallel to the fiber axis) and tetramer-like (the nucleosome axes are at 45° with respect to the fiber axis and mutually perpendicular as found in the x-ray tetramer structure [3]). In our convention, the trans conformations correspond to $\eta=-25°$ or $-25+180$ and the relative helical structures are characterized by $\psi=130°$; the cis conformations correspond to $\eta=-115°$ or $-115+180$ and the relative helical structures are characterized by $\psi=-50°$, and the tetramer conformations correspond to $\eta=-70°$ or -70 + 180 and the relative helical structures are characterized by $\psi=40°$ (see Fig. 3A). The conformational parameter ψ allows the evaluation of the change of twist angle per bp, Ω, and the corresponding torsional energy required to obtain a given helical class with a linker length, *l*. Adopting a first order elastic model:

$$E = \frac{k_{tw}(\Delta\Omega)^2}{2l} \quad \text{where} \quad \Delta\Omega = \cos^{-1}\left[\cos\left(\psi(\eta)-\frac{360l}{10.5}-15\right)\right] \quad (6)$$

$360° \, l/10.5$ is the DNA linker twist pertinent to *l* bp, adopting the DNA helical periodicity of 10.5 bp per turn as the relaxed twist. The value of 15° in Eq. (6) corresponds to the DNA entry–exit twist difference of the nucleosome core as evaluated from the x-ray structure [1]. In fact, if two nucleosomes are hypothetically joined together, namely $l=0$, the twist angle to obtain the standard B-DNA structural continuity ($\Delta\Omega=0$) is 15°. k_{tw} is the torsional elastic constant and the value of 0.0274 kcal·bp^{-1}·deg^{-2}, corresponding to 90 kcal·bp^{-1}·rad^{-2}, is adopted as an average value that accounts for DNA circularization experiments ([25] and references therein). The operator $\cos^{-1}\cos$ reduces the modulus of the torsion angle to its minimum value within 180° since the quadratic dependence of the torsional energy on the twist deviations produces equivalent values if the DNA linker is twisted or untwisted with respect to the relaxed state.

Fig. 3B (shown below 3A and C) illustrates the torsional energy profiles reported as a function of NRL between 150 and 240 bp for trans, cis and tetramer conformations. This diagram is representative of the fiber structures characterized by nucleosome helical axis nearly perpendicular, parallel or tilted with respect to the fiber axis, independently of the helical class. The torsional energy changes periodically with a period of 10.5 bp for all the conformations. The energy changes versus the linker length are rather large for short NRLs; as a consequence, the helical conformations are very constrained. If a different twist periodicity for the linker is adopted, e.g. 10.4 bp, the torsional energy and phase of short linkers do not change significantly. However, progressive phase changes with the lengthening of linkers are introduced.

Fig. 3C illustrates the profiles of torsional energy for trans, cis and tetramer-like conformations for the particular NRLs between 177 and 237 bp with steps of 10 bp corresponding to the reconstituted nucleosome arrays investigated by Robinson et al. [4]. It should be

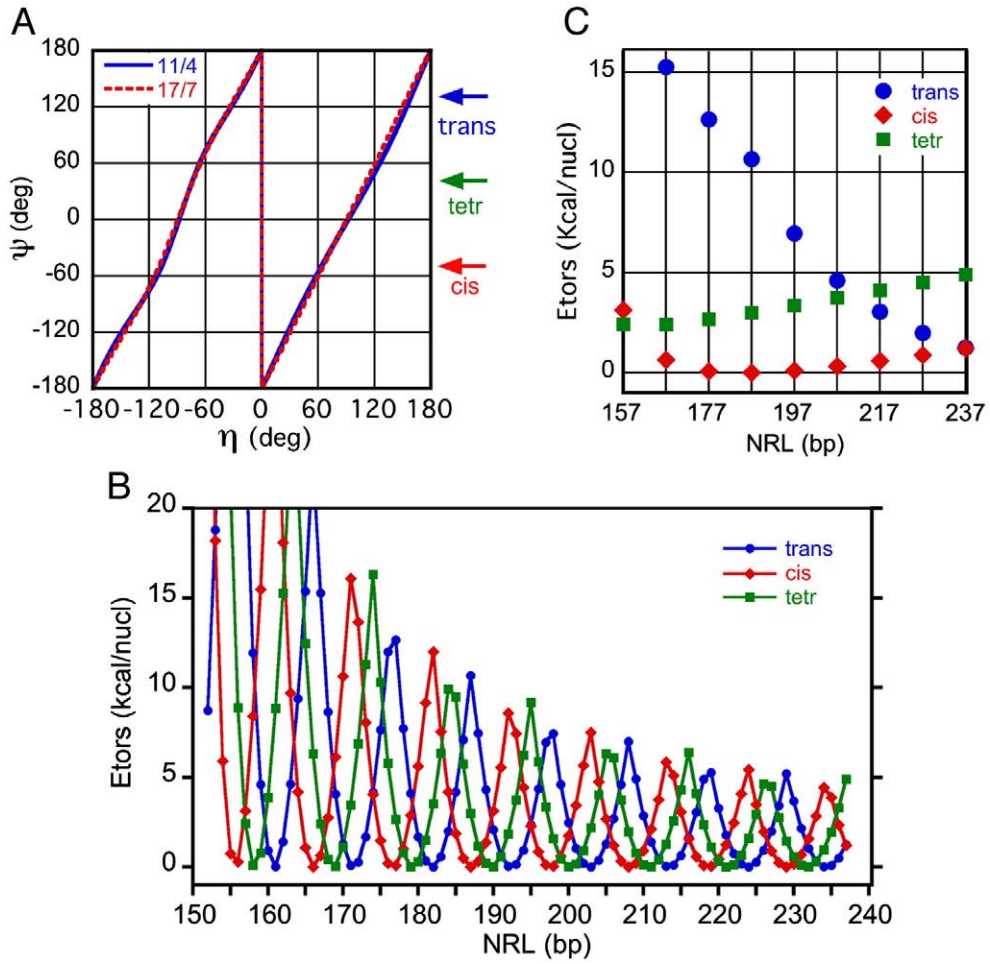

Fig. 3. Torsional angle ψ as a function of nucleosome tilt angle η and torsional energy calculated in terms of angle ψ. (A) Angle ψ versus the nucleosome tilt angle η in the cases of the 11/4 (blue line) and 17/7 (red line) helix classes, shown as examples. Trans-like, cis-like and tetramer-like conformations are defined as in the text. In our convention, the trans conformations correspond to $\eta = -25°$ or 155° ($\psi = 130°$); the cis conformations correspond to $\eta = -115°$ or 65° ($\psi = -50°$), and the tetramer conformations correspond to $\eta = -70°$ or 110° ($\psi = 40°$); (B) torsional energy profiles as a function of nucleosome repeat length between 150 and 240 bp for trans, cis and tetramer conformations. The torsional energy is calculated adopting the relaxed DNA linker twist of 360/10.5 and a torsional elastic constant equal to 90 kcal·bp^{-1}·rad^{-2}; (C) torsional energy profiles for trans, cis and tetramer-like conformations in the particular cases of NRL between 177 and 237 bp with steps of 10 bp corresponding to the reconstituted nucleosome arrays investigated by Robinson et al. [4]. In spite of the apparent trend continuity, the torsional energy reported in terms of NRL is not continuous as illustrated in Fig. 3B.

noted that, in spite of the apparent trend continuity, the torsional energy reported in terms of NRL is not continuous as clearly illustrated in Fig. 3B but only pertinent to the NRL in the abscissa. For relatively short NRLs, the torsional energy is particularly high for the trans conformations, where the nucleosome helical axes are nearly perpendicular to the fiber axis. In this case, the steep increase in the torsional energy should favor cis conformations. If the linkers are changed by 5 bp, about a half DNA turn, the profiles of torsional energy for the trans and cis conformations interchange (see Fig. 3B). In this regard, different authors [26–28] found a linker length periodicity of $10i + 5$, (i is an integral number) on chromatin of different cells and species that would favor the trans conformations.

2.4. Nucleosome core packing in compact chromatin fibers

Packing of nucleosome cores in chromatin fiber was investigated by several authors adopting the Gay–Berne or Zewdie potential equivalent to a Lennard-Jones potential with ellipsoid symmetry [10,29] or a spherocylinder symmetry [11,12].

A different strategy is adopted in this paper. The packing of nucleosomes in compact structures is simulated by representing the nucleosome as a simple parallelepiped that fits the external nucleosome surface (see Fig. 4A) in order to select the excluding-volume structures. These are refined in the final step adopting a Lennard-Jones-like potential function, as will be explained later.

The packing distance between the adjacent nucleosomes is evaluated in terms of the rotation angle η for all the helical classes with n ranging between 6 and 17 (Fig. 4A). Such a distance has an inferior limit of around 4.0–5.0 nm, about the axial dimension of nucleosome core. The helical arrangement of nucleosome cores on the external cylindrical surface shows a pitch increasing with a parameter, m, which changes with the helix class and indicates the number of nucleosomes between the nearest-neighbor nucleosome units in the fiber following the DNA chain (see Fig. 2A). The packing distance, pd, was evaluated as the distance between the planes perpendicular to the nucleosomal DNA helical axis $\mathbf{h}_n$, containing its dyad axis at the radial distance (R) of the nucleosome helical axis (the fiber radius minus 5.5 nm) (see Fig. 4A) as:

$$pd = abs\left\{\left[(2R\sin(\phi m/2))^2 + (11m/n)^2\right]^{\frac{1}{2}} \times \cos\left[\tan^{-1}\left(\frac{11m/n}{2R\sin(\phi m/2)}\right) - (\eta + 25)\right]\right\} \quad (7)$$

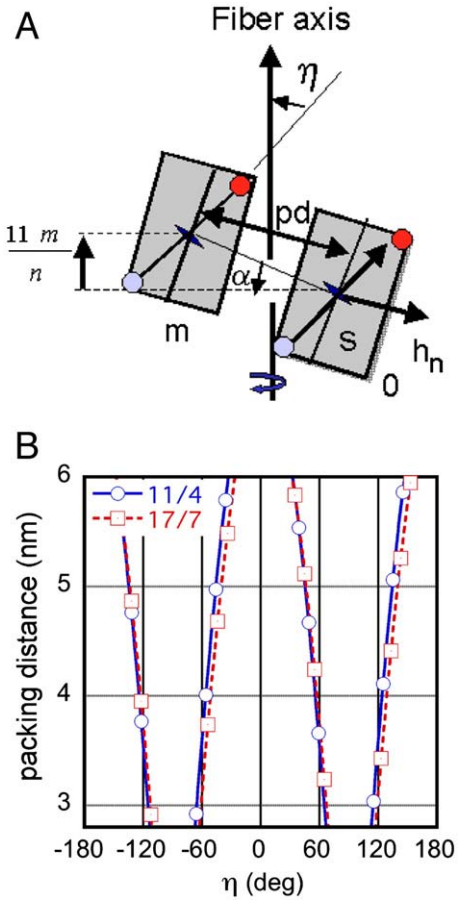

Fig. 4. Scheme of packing distance and the relative profiles in terms of the nucleosome tilt angle. (A) Packing distance between the adjacent nucleosomes evaluated as the distance between the planes perpendicular to the nucleosomal DNA helical axis $\mathbf{h}_n$, containing its dyad axis at the radial distance (R) of the nucleosome helical axis. The sign of m determines the chirality of the arrangement of nucleosome cores along the fiber: if positive, the apparent nucleosome core helix is right-handed independently of the chirality of the linker chain; (B) packing distance profiles versus the angle η for two representative right-handed helix classes: 11/4 (blue line) and 17/7 (red line). 34 and 44 nm were adopted as fiber diameters according to the experimental findings for n equal to 11 and 17, respectively [4]. For the corresponding left-handed helices (t negative), the profiles become the mirror image across a plane at $\eta = 25°$. The arrows indicate the allowed fiber structures compatible with the packing and the corresponding crossing distances (Fig. 2B).

The sign of m determines the chirality of the arrangement of nucleosome cores along the fiber: if positive, the nucleosome core helix is right-handed independently of the chirality of the linker chain. Therefore, the chiral arrangement of nucleosomes can be coherent or anti-coherent with that of the linker chain.

Fig. 4B illustrates the change of packing distance versus the angle η for the right-handed helix classes 11/4 and 17/7 chosen as examples. The values 34 and 44 nm are adopted as the fiber diameters according to the experimental findings for n between 11 and 14 and 15 and 17, respectively [4]. Alternative choices result in loose or clashing of nucleosome packing. For the corresponding left-handed helices (t negative), the profiles in Fig. 4B become the mirror image across a plane at $\eta = -25°$ which defines the fiber structures characterized by the nucleosomal DNA helix axis perpendicular to that of the chromatin fiber. The packing diagrams show a translational symmetry of 180° due to the nucleosome dyad symmetry. However, this symmetry does not hold for the linker chain as can be seen in Fig. 1B. As a matter of fact, the corresponding structures, in spite of the equivalent nucleosome packing, should be topologically different if the integral twist is conserved. A rotation of $\pm 180°$ around the nucleosome dyad axis produces the same nucleosome packing but a twist change of DNA linker equal to $\pm 360°$ is required (see Fig. 3A).

It is worth noting that taking into account the diagram of Fig. 2B, which localizes the allowed values of η to avoid the clash of linkers (indicated by arrows), only one fiber structure is compatible with the close packing of nucleosomes for the 11/4 helix and two for 17/7 helix (arrows in Fig. 4B). Packing distance diagrams were evaluated for all the helical classes.

The compact nucleosome packing in chromatin implicitly determines the range of the linker length. In fact, the actual linkers are constrained by their projection on the straight virtual linkers selected to fit the axial and radial nucleosome density. This can be obtained by curving the DNA linker about its virtual axis. Indeed, the DNA linkers show a little curvature in the x-ray crystal structure of the tetranucleosome obtained by Schalch et al. [3]. Nevertheless, the fiber structures defined with virtual linkers are an adequate approximation of the essential features of the chromatin fiber.

3. Results and discussion

Geometrical features, which characterize the different helical classes with close packing of nucleosome residues, severely limit the possible chromatin structures. In this paper, we show that besides the geometrical restraints also topological and conformational features are important parameters for selecting the fiber compact structures under the realistic assumption of the structural conservation of the nucleosome core and minimum distortions of DNA linkers.

Compact fiber architectures are mainly stabilized by the close packing of nucleosome cores topologically constrained to prevent linker chains from clashing and entangling. Furthermore, the geometrical conditions, which determine the best packing of the nucleosomes, require a torsional energy cost to account for the changes of DNA linker twist, particularly stringent for short linkers. Such an energy cost represents the main contribution in the regulation of the shape and flexibility of chromatin open conformations. The phasing angle between DNA linker at the exit of a nucleosome and the entry of the consecutive one selects the fiber structure, due to the natural DNA helical twist as Wong et al. [15] also emphasized.

Stacking between the larger surfaces of nucleosomes controls the stability of the fiber in the case of relatively low axial density of nucleosomes as in the two-start helices where the nucleosome interactions along the fiber axis are negligible. Fig. 5 shows the crossing and packing distances, the torsional angle and relative torsional energy for the chromatin fiber in the case of two-start helices. These are only allowed for relatively low axial densities of the chromatin fiber as already discussed, since the linker crossing close to the fiber axis requires a distance equal to two times the axial repeat to avoid their clashing. In the figure, we adopted the highest allowed nucleosome density (about 8 nucleosomes per 11 nm), nucleosome repeat length equal to 177 bp and a fiber diameter of 30 nm. These values are consistent with two-start helices belonging to the helical class 13/6.

Matching the allowed crossing distances with the best packing distances, two structures are obtained: a right-handed and a left-handed helix of the linker chain at η about $-70°$ and $+10°$, respectively (see Fig. 5A and B). Another right-handed helix can be obtained at $\eta = -20°$ ($pd = 3.7$ nm) but a lower axial density, about 7 bp per 11 nm, is required. The right-handed structures are characterized by different conformational angles: ψ about 50° and 135° (adopting our convention), corresponding to those of the crystal structure of the tetranucleosome [3] and to the trans conformation [4], respectively (Fig. 5C). The latter conformation requires a larger torsional stress of linkers corresponding to about 13 kcal/nucl (see Fig. 5D), 10 kcal/nucl more than that relative to the tetramer-like conformation. The left-handed structure is characterized by a conformational angle $\psi = 135°$. This conformation, however, requires a relatively large distortion of the linkers as can be deduced from the energy profiles in Fig. 3B and C.

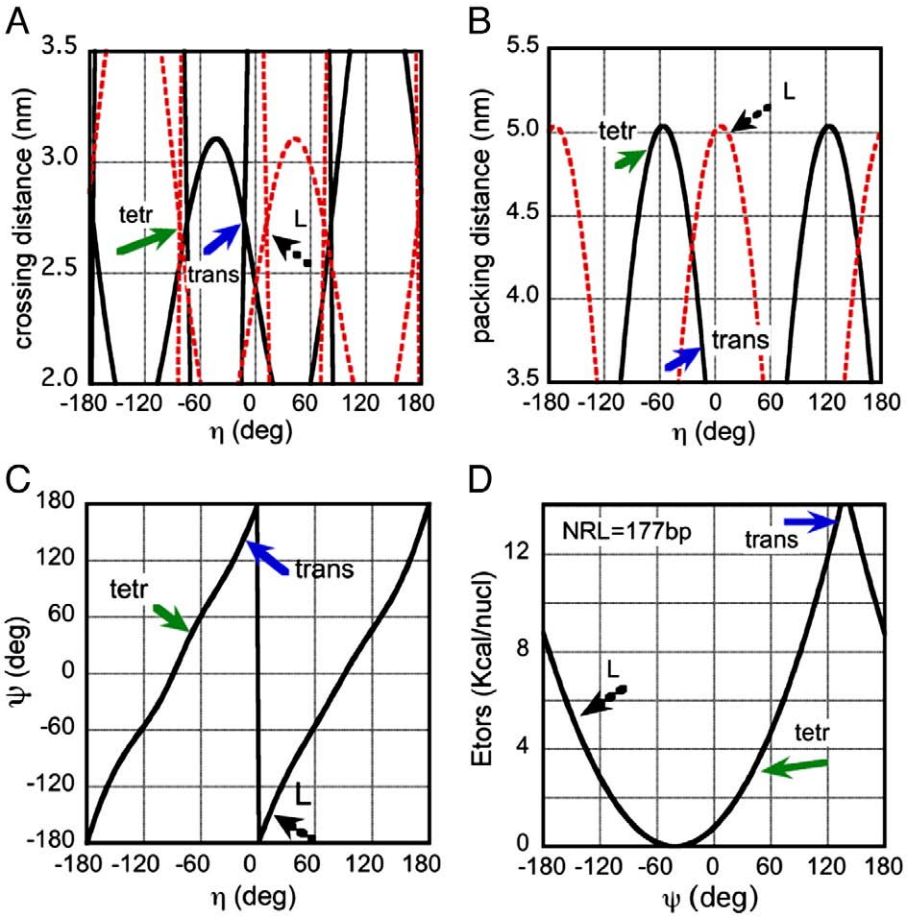

Fig. 5. Diagrams of crossing distances, packing distances, torsional angle and torsional energy. The diagrams refer to two-start helix conformations compatible with the helical class 13/6, NRL of 177 bp, a fiber diameter of 30 nm, and the highest allowed nucleosome density of about 8 nucleosomes per 11 nm. (A) Crossing distances as a function of the tilt angle η. The profile relative to the crossing distance between $l_{ex}(0)$ and $l_{en}(0)$ is very similar to that in Fig. 2B and not reported for the sake of clarity. However, it was considered to select the possible conformations; (B) profiles of packing distances as a function of the tilt angle η. Black lines are for right-handed helices, red lines for left-handed; (C) torsional angle ψ as a function of the tilt angle η; (D) torsional energy as a function of the torsional angle ψ. Matching the allowed crossing distances with the best packing distances, three structures are possible (indicated by the arrows). Among these three conformations, the trans structure requires the largest torsional stress of the linkers (Fig. 5 D).

Fig. 6 shows the two structures characterized by nucleosomes core packing that follow a left-handed helix whilst the linker chain chirality is right-handed. They were obtained after an appropriate refinement of the conformational energy by minimizing the interactions between the pseudo-atom centers, which fit the positions of phosphate groups in their projection on the DNA axis. A Lennard-Jones-like potential function was adopted with the minimum positioned at 2.5 nm, which corresponds to the closest distance between B-DNA axes [24]. These structural models are very similar to those proposed by Schalch et al. [3] and virtually capable of forming the cystine bridges between the C-mutant histones as in the experiment by Dorigo et al. [6].

In the case of high axial density of nucleosomes the stability of the chromatin fiber requires more complex helical structures, where the packing of nucleosomes involves the adjacent nucleosomes, mth and $(n–m)$th units along the helix, with $m>2$.

For the dense structures in the case of NRLs between 177 and 237 bp with steps of 10 bp, the actual linkers were obtained by curving the DNA axis about its virtual axis; this deformation from the straight DNA structure does not sensitively change the twist angle. As shown in Fig. 3C, trans conformations allow close packing of nucleosomes but require significant torsional stress of DNA linkers for nucleosome repeat length equal to $147+5\cdot i$ bp, i even, mainly for the shorter NRLs. However, increasing the nucleosome repeat length reduces the torsional stress with the lengthening of the DNA linker and could justify the transition of the chromatin fiber diameter and density as EM images demonstrate [4]. Despite the cis conformations are torsionally more relaxed, the nucleosome packing is less effective due to the wedge shape of the nucleosome structure (see Fig. 1); consequently, the radial packing is favored with respect to the columnar one of the cis conformations.

Some relevant fiber architectures are illustrated in Fig. 7, obtained after an appropriate refinement of the conformational energy as described in the paragraph 2.4.

Fig. 7 illustrates how increasing the linker length by steps of 10 bp induces the rotation around the nucleosome dyad axis. The tilt angle progressive changes result in the reduction of the central hole and, as a consequence, in quasi-invariance of the fiber diameter (~33 nm) of the 13/5 interdigitated helical structures (see the structures for 177, 197 and 207 bp NRLs). A further increase in the linker length induces a transition to a different 17/5 helical class characterized by a larger diameter (~45 nm) and tilt angle shifted by 180°. Such a rotation around the nucleosome dyad axis preserves the same packing of nucleosome units. It is plausible that different entry–exit linker regions and arrangements of linker histones characterize the two structural classes. These fiber structures appear fully consistent with EM experiments by Robinson et al. [4]; furthermore, they show minimum distortions of the intrinsic nucleosome and linker structures.

However, a systematic search of the possible fiber architectures reveals the existence of other structures. Fig. 8 shows two fiber

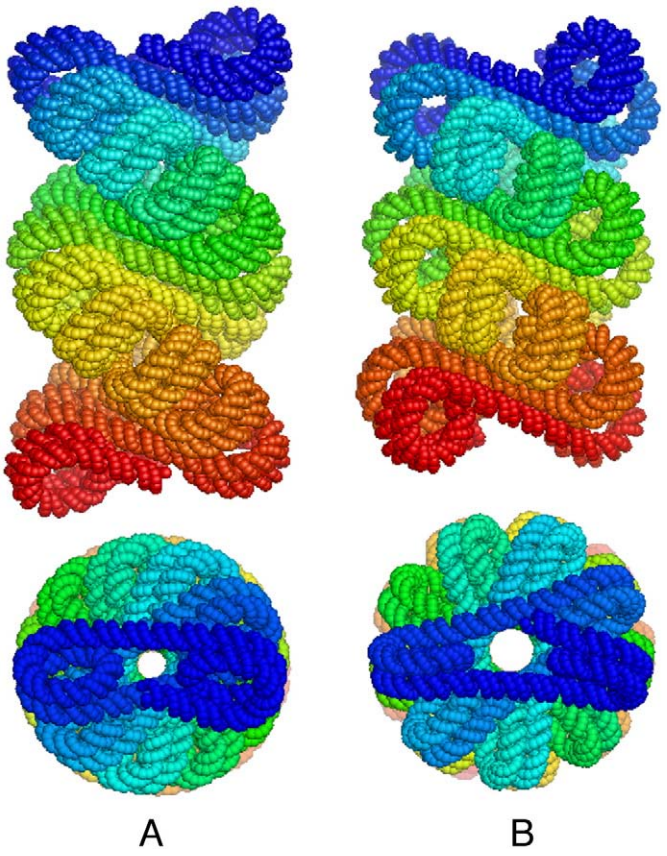

Fig. 6. Structural models for two-start helices with 177-bp nucleosome repeat length. The two structures belong to the helical class 13/6 with 177-bp NRL, and a fiber diameter of 30 nm and are characterized by left-handed nucleosome core packing whilst the linker chain chirality is right-handed. (A) Helical structure with a tetramer-like conformation with the features indicated by the green arrow in Fig. 5A–D; (B) helical structure with a trans-like conformation with the features indicated by the blue arrow in Fig. 5A–D. The nucleosome packing ratio is ~5.5 nucleosomes/11 nm equivalent to 0.14 g/ml for both the structures. The structures are shown in axial and azimuthal projections.

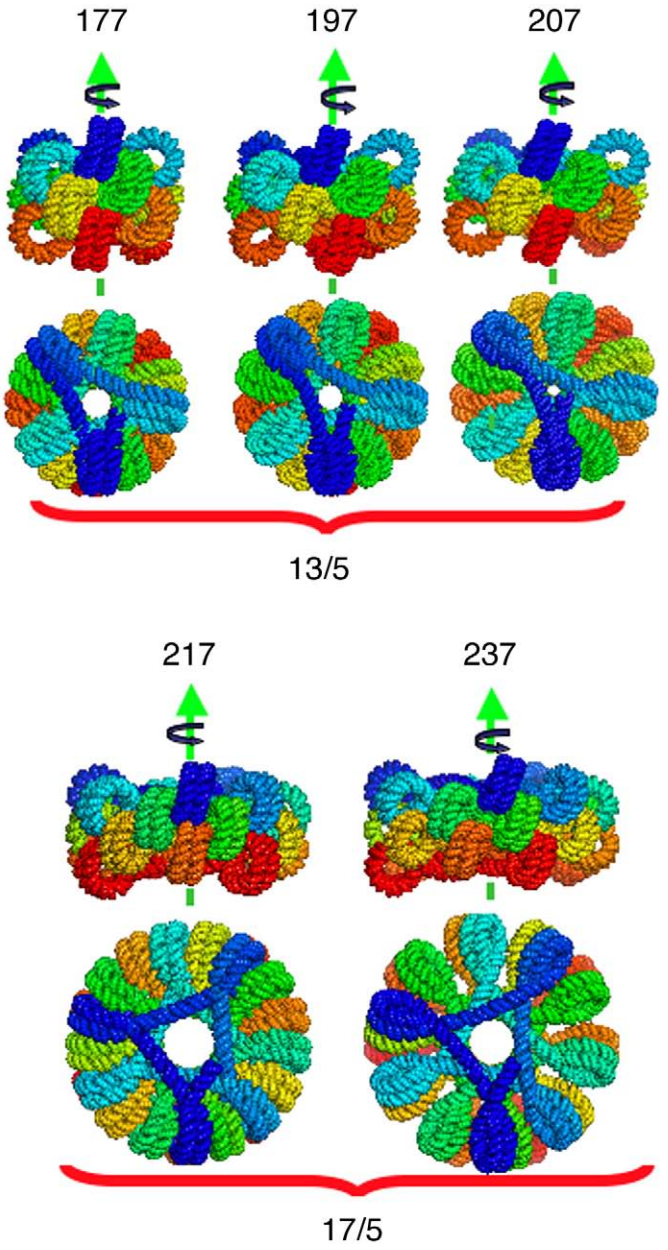

Fig. 7. Models for interdigitated structures. Helical structures selected for 177, 197, 207, 217 and 227 bp nucleosome repeat lengths after calculating packing and crossing distances in terms of the tilt angle η and refining. The first three right-handed structures (helical class 13/5) are characterized by interdigitated structures and a fiber diameter of ~34 nm (nucleosome packing ratio ~11 nucleosomes/11 nm equivalent to 0.23 g/ml). A further increase in the linker length determines a transition to an interdigitated 17/5 helical class characterized by a larger diameter (~45 nm) and a tilt angle shifted by 180° (nucleosome packing ratio ~15 nucleosomes/11 nm equivalent to 0.22 g/ml). Axial and azimuthal projections are reported.

architectures, alternative to the interdigitated structures reported in Fig. 7. The helical class 13/4 is shown in Fig. 8A for 177 bp NRL; it can be considered a three-start helix due to the nucleosome packing consistent with the x-ray crystal structure of the tetranucleosome [3]. The linker chain is right-handed whilst the nucleosome core packing is characterized by a left-handed chirality. This structure, however, is not suitable for all NRLs ranging from 177 to 207 bp, since the fiber diameter would be too large to match the experimental values [4]. As a consequence, DNA mass density would be too low. A thinner fiber can be obtained with structures belonging to the class 12/-5, as that reported in Fig. 8 for 207 bp NRL. It should be noted that this structure shows the same left-handed chirality of the linker chain and nucleosome core packing, in order to avoid steric clashes among the DNA linkers, as revealed by the crossing distance calculations for this helical class. Incidentally, this helical class corresponds to the (-5,7) interdigitated model proposed by Staynov and Proykova [17] to account for digestion experiments that prove the non-sequential arrangement of nucleosomes along the fibers.

The chromatin fibers with NRLs between 217 and 237 bp can assume different conformations since a relatively small torsional energy is required (see Fig. 3B and C). In fact, the trends of torsional energy show an inversion between tetramer-like and trans conformations for nucleosome repeat length between 207 and 217 bp. Therefore, the transition in the axial nucleosome density and diameter of the chromatin fiber, evidenced by EM images [4], could be due to the necessity to minimize the torsional stress of linkers.

After refining the conformational energy, other fiber architectures are obtained for 217–237-bp nucleosome repeat lengths. Four possible helix classes are proposed for a 227-bp nucleosome repeat length (chosen since intermediate in the range 217–237 bp) and ordered by increasing the cylindrical repeat angle in Fig. 9: 17/5, 16/5, 17/6, and 17/-7. This last class, although characterized by the left-handed chirality of the linker chain, shows the nucleosome core packing arrangement consistent with the cross-linking experimental results obtained by Dorigo et al. [6]. The nucleosome packing ratio and fiber diameter of all the chromatin fibers in Fig. 9 are consistent with the experimental data obtained by EM visualizations [4].

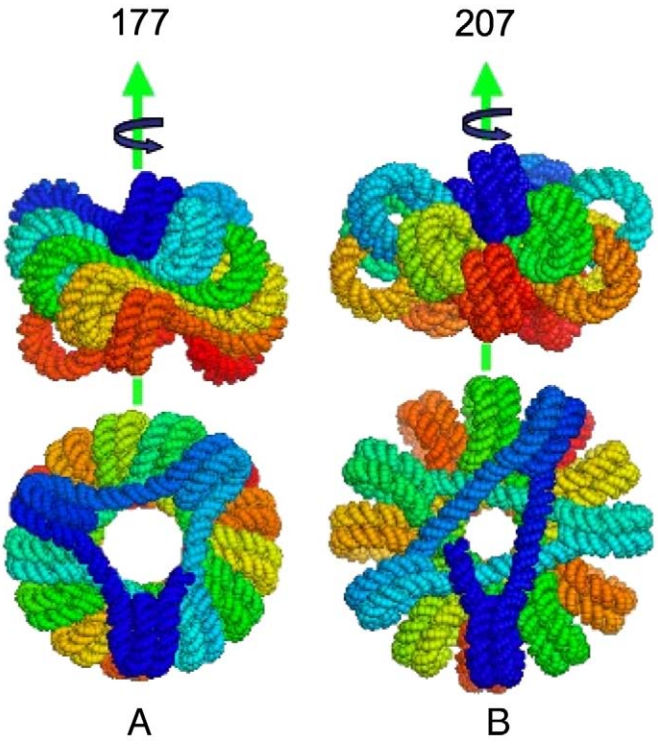

Fig. 8. Alternative structural models for 177–207 bp nucleosome repeat lengths. Two fiber architectures, alternative to the interdigitated structures shown in Fig. 7, for 177 and 207 bp nucleosome repeat lengths. (A) Right-handed structure belonging to the helical class 13/4 for 177 bp NRL (nucleosome packing ratio~10.5 nucleosomes/11 nm equivalent to 0.21 g/ml); (B) left-handed structure belonging to the interdigitated helical class 12/-5 for 207 bp NRL (nucleosome packing ratio~10.6 nucleosomes/11 nm equivalent to 0.22 g/ml). The structures are shown in axial and azimuthal projections.

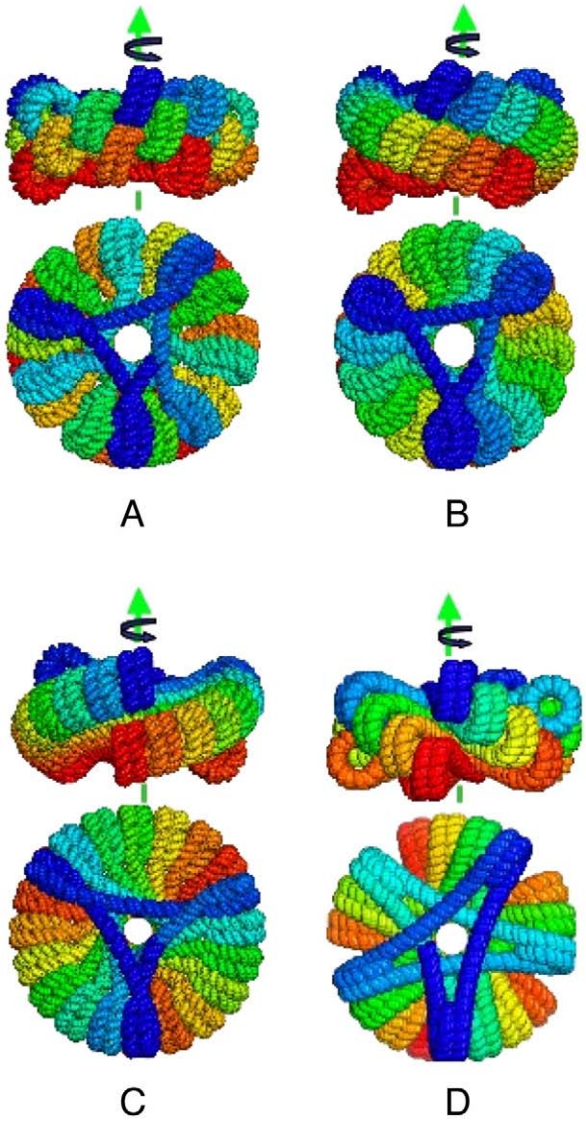

Fig. 9. Alternative structural models for 217–237 bp nucleosome repeat lengths. Different helical structures allowed for the chromatin fiber with nucleosome repeat length of 227 bp: (A) right-handed helix structure belonging to the class 17/5 (nucleosome packing ratio ~15.7 nucleosomes/11 nm equivalent to 0.23 g/ml); (B) right-handed helix structure belonging to the class 16/5 (nucleosome packing ratio ~15 nucleosomes/11 nm equivalent to 0.22 g/ml). (C) Right-handed helix structure belonging to the class 17/6 (nucleosome packing ratio ~15.5 nucleosomes/11 nm equivalent to 0.23 g/ml); (D) left-handed helix structure belonging to the class 17/-7 (nucleosome packing ratio~15 nucleosomes/11 nm equivalent to 0.21 g/ml). The structures in B and D have the nucleosome core packing arrangement consistent with the cross-linking experimental results obtained by Dorigo et al. [6]. The structures are shown in axial and azimuthal projections.

A narrow range of entry–exit angles characterizes all the models of fiber structures proposed for the same nucleosome repeat length; therefore, all of them are plausible candidates to interact with the unstructured tails of linker histones. They are also characterized by different dimensions of the central cylindrical hole that could accommodate various proportions of linker histone as an interstitial moiety, which contributes to neutralize the DNA linker electrostatic repulsions of more nucleosome repeat units [30].

It is interesting to note that all the structures selected for 177–237 bp NRLs (Fig. 7) are characterized by left-handed nucleosome core packing according to the electron microscopy Fourier transform of helical tracts of natural chromatin fibers reported in the pioneering paper by Williams et al. [31].

A relevant parameter to evaluate the different structural models proposed for the compact chromatin fibers is the concentration of DNA in the fiber volume (given as g/ml) [32]. Indeed, chromatin fibers with similar diameters and nucleosome packing can accommodate linkers with different lengths and curvature, which regulate the DNA packing density. The models of chromatin fiber we propose have values of linear packing ratio within the range 0.15–0.23 g/ml equivalent to 6–16 nucleosomes/11 nm. The lowest value refers to the structural models (see Fig. 6) very similar to those proposed by Schalch et al. [3] and compatible with the corresponding experimental density. The highest values are relative to the interdigitated structures proposed for the 177–237 bp NRLs and in good agreement with the experimental densities by Robinson et al. [4].

The extension of our analysis on the compaction of regular arrays of nucleosomes to natural chromatin is obviously limited to DNA tracts that show regular repeating sequence for which the principle of conformational equivalence can be assumed. Among these, the telomeric chromatin is particularly interesting due to the short periodicity of the sequence that produces a large degeneration of nucleosome positioning favoring helical structures of the fibers plausibly regulated by the concentration of linker histone [30].

However, packing of the nucleosomes in chromatin fiber requires some regularity and phasing. Genomes in vivo are often characterized by uniformly spaced positions of nucleosomes needed to fold chromatin fibers. In this respect, several experimental results showed that the relative uniformity of the nucleosome distribution is the major feature of different genomes. As a matter of fact, genome-wide nucleosome positioning maps reveal a repeat length of 165 bp for *Saccharomyces cerevisiae* [33], 175 bp in the *Caenorhabditis elegans* [34], about 200 bp along human genome [35] and 154 bp for *Schizosaccharomyces pombe* [36].

Moreover, the analysis of genomic DNAs shows sequence signals in phase with the DNA helical periodicity that suggests a regular nucleosome positioning [26–28]. Phasing of nucleosome positioning along DNA drives the nucleosome packing in chromatin. Genome regions where the nucleosomes are positioned in phase (e.g. nucleosome repeat length equal to $147+5\cdot i$ bp, i even) are torsionally relaxed in chromatin fiber characterized by nucleosome helical axes quasi-parallel to the fiber axis, whilst the perpendicular arrangement should be favored in the case of nucleosome dyad axis positioning out of phase (e.g. nucleosome repeat length equivalent to $147+5\cdot i$ bp, i odd). In this respect, some interesting information emerges from theoretical models predicting nucleosome positioning on one-dimensional DNA sequence based on the periodical distribution of particular sequence signals phased with the DNA periodicity [28,37], or on the theoretical evaluation of the differential free energy of nucleosomes along the DNA sequence [38].

It is interesting to note that the relative phase of nucleosome dyad axis positioning with respect to that of the adjacent one along DNA, $\Delta phase$, is related to the DNA linker twisting change by:

$$\Delta\Omega = \cos^{-1}\left[\cos(\psi(\eta)-\Delta phase-15)\right] \quad (8)$$

The strongly positioned nucleosomes along a multimeric Widom 601 sequence [5] cooperate in stabilizing a helical architecture of the chromatin fiber on the basis of the principle of conformational equivalence of the repeating units. For the natural chromatin, it is plausible that the presence of strongly positioned nucleosomes could act as a nucleation step inducing equivalent arrangements on those weakly positioned to favor proper packing.

The analysis reported in this paper shows that geometrical, conformational and topological conditions strongly constrain the possible compact architectures of the chromatin fiber. The close packing of nucleosomes represents the main contribution to the stability of the chromatin fiber, which generally involves non-consecutive nucleosome units along DNA according to the DNase I digestion pattern [16,17]. Furthermore, we demonstrated that the experimental data of electron microscopy can be reproduced without strongly perturbing the nucleosome and DNA linker intrinsic structures [4].

Importantly for determining the different kinds of nucleosome packing it is the torsional energy involved that appears more critical for short NRLs as in telomeres, yeast and neuronal chromatin. It is worth noting that the chirality of nucleosome packing is independent of that of the DNA linker chain that can be left- or right-handed although retaining nucleosome packing as shown in Fig. 9. Therefore, the change of the twisting number of the chromatin fiber in its writhing transition from a compact to an open state is the sum of the opposite contributions of the right- and left-handed linker chain, which reduces the topological constrains of the relative invariance of the linking number as well as the torsional energy involved.

The images of all the structures shown in Figs. 6–9 were realized with the PyMOL Molecular Graphics System [39].

Acknowledgements

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