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REVIEW

Structure and Functions of Linker Histones

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Abstract—Linker histones such as variants H1, H5, and other similar proteins play an important role in regulation of chromatin structure and dynamics. However, interactions of linker histones with DNA and proteins, as well as specific functions of their different variants, are poorly studied. This is because they acquire tertiary structure only when interacting with a nucleosome, and because of limitations of currently available methods. However, deeper investigation of linker histones and their interactions with other proteins will address a number of important questions – from structure of compacted chromatin to regulation of early embryogenesis. In this review, structures of histone H1 variants and its interaction with chromatin DNA are considered. A possible functional significance of different H1 variants, a role of these proteins in maintaining interphase chromatin structure, and interactions of linker histones with other cellular proteins are also discussed.

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Elucidation of structural organization of genetic material in the interphase nucleus is one of the most important problems in understanding of gene expression. Although the structure of the nucleosome core particle was determined [1], structural features of higher levels of chromatin compaction remain a subject of much controversy. It is well established that linker histones play a pivotal role in formation and stabilization of 30-nm chromatin fibril structure.

All variants of these histones have similar structure. They consist of approximately 200 a.a. and are organized into three structural fragments: N- and C-terminal tails that are unstructured in the absence of nucleosomes, and a globular central domain (Fig. 1).

Histone H1, one of key chromatin structural proteins, also called a linker histone, has been known for a long time. Nevertheless, its structure and location in chromatin (in contrast to nucleosome core histones) are studied insufficiently.

The location of histone H1, which is not contained in the nucleosome core but apparently contributes to assembly of chromatin higher-order structures, namely 30-nm fibrils, is still being debated. The most probable location of this protein is a region close to the symmetry axis of the nucleosome that is situated in the vicinity of DNA entry and exit sites in a nucleosome particle (Fig. 2). This supposition is supported by results of both molecular biological experiments [2, 3] and studies performed by computational techniques [4, 5]. The precise arrangement of a linker histone in the chromatosome (nucleosome core particle bound to a linker histone) remains obscure.

BRIEF CHARACTERISTICS OF STRUCTURE OF LINKER HISTONES

The globular domain of linker histones has a winged helix structure comprising a helix-turn-helix motif [6, 7]. The α-helices in the globule are designated as H1, H2, and H3 (counting from N-terminus proximal α-helix) [5], or as α1, α2, and α3 [8] (Fig. 1). The globular domain of linker histones can independently bind to the nucleosome *in vitro* [2, 3]. The winged helix fold is widespread among DNA-interacting proteins [9]. Proteins having a similar fold are found in eubacteria, while core histone counterparts are found only in Archaea [10]. This suggests that core and linker histones were acquired by

Abbreviations: NMR, nuclear magnetic resonance.
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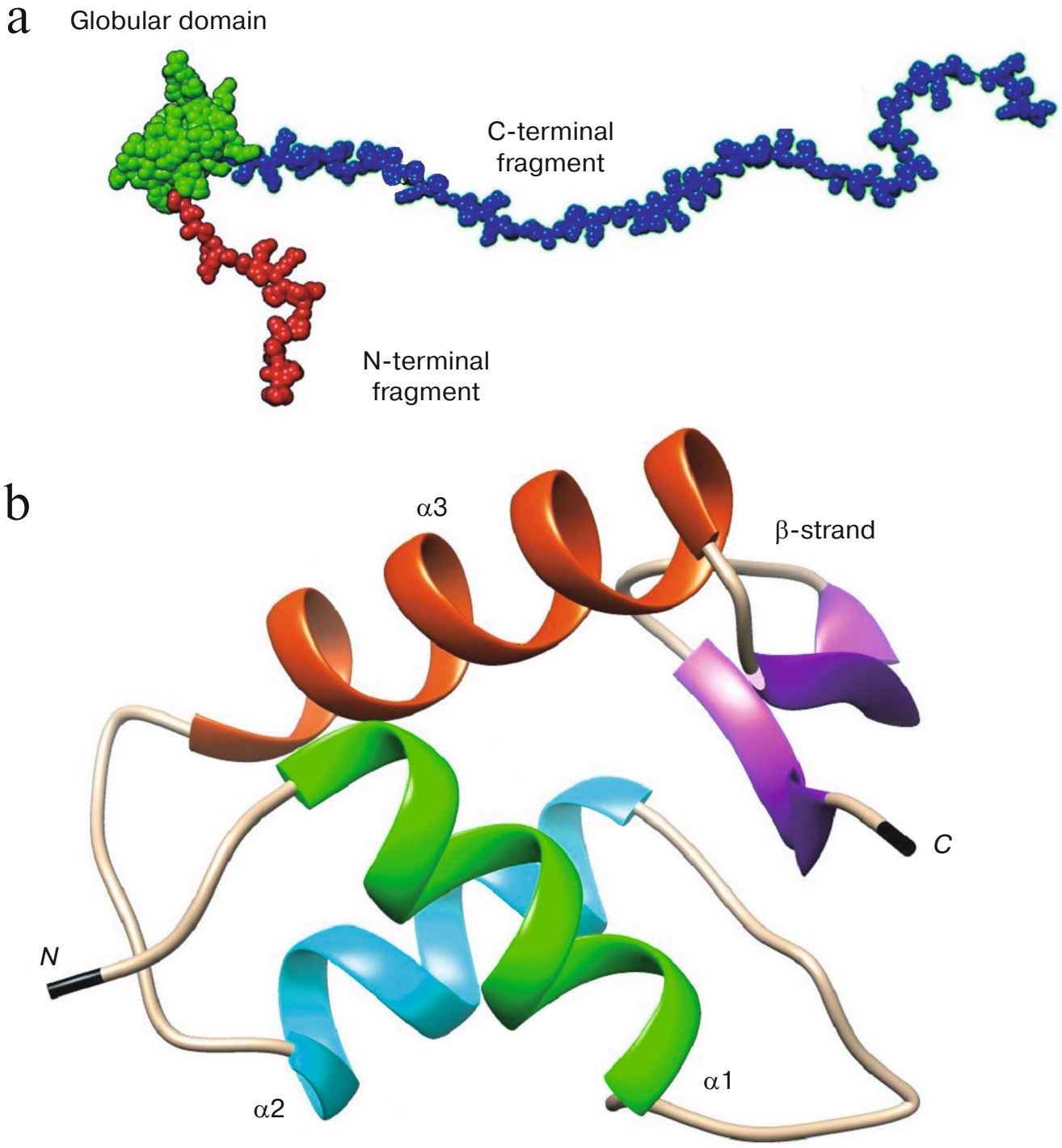

Fig. 1. Structure of a linker histone. When the N- and C-terminal fragments of the linker histone do not contact the nucleosome, they are unstructured. The globular domain located between them consists of three α-helices (α1, α2, and α3; counting from the N-terminus) and a small β-strand, which is called a "wing".

eukaryotes independently from each other in the course of evolution [11].

Almost the entire surface of linker histones is positively charged, in contrast to the majority of proteins having a winged helix fold that possess dipole moment [5]. At the same time, nonhistone proteins with such fold have only one DNA-binding site, while there are at least two such sites on linker histone globules, though their precise location is still not established [8, 12, 13]. All proposed models of arrangement of linker histone in the nucleo-

some also presume existence of at least two contacts with DNA [8, 14]. Macromolecular docking studies [5] and other quantitative methods [4] support this point of view and suggest that the histone H5 globule can form three contacts with DNA and interacts with the minor groove of DNA in the vicinity of the nucleosome symmetry axis. Hydroxyl radical footprinting studies [3] also support this conclusion. At the same time, the number of contacts between the linker histone globule and DNA apparently depends on condensed chromatin geometry and, particularly, on the length of the linker fragments between nucleosome particles [5, 15].

Until recently, it was accepted that nucleosomal DNA in the vicinity of the symmetry axis on the nucleosome was contacted by helix H3 of the globular domain of H1 [4, 5]. Recent NMR studies with paramagnetic labels [8] also favor this interaction model. As it was considered earlier, linker DNA was contacted by amino acid residues of helices H2 and H3 [5] or residues of the β-strand and loops adjacent to the C-terminus of the H1 molecule [4] (Fig. 3). At the same time, examination of point mutation effects on the H1 globule binding to the nucleosome contradicts participation of these fragments in interaction with linker fragments [8]. According to a model built in this study, a surface interacting with linker DNA is formed exclusively by amino acids of helix H2 and loops between elements of ordered secondary structures (Fig. 3). Moreover, there are data indicating that the

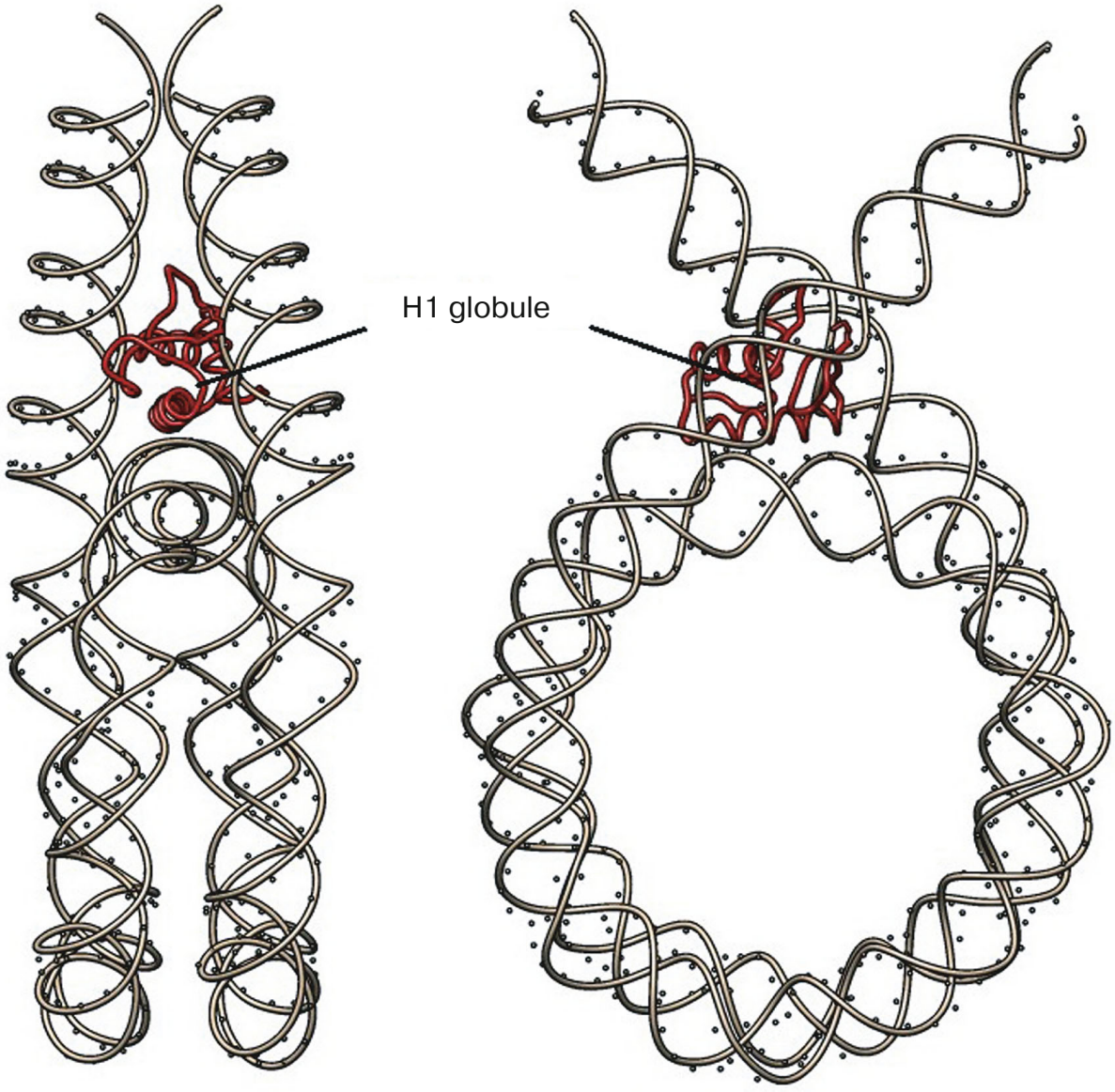

Fig. 2. Arrangement of the globular domain of linker histone H1 on the nucleosome.

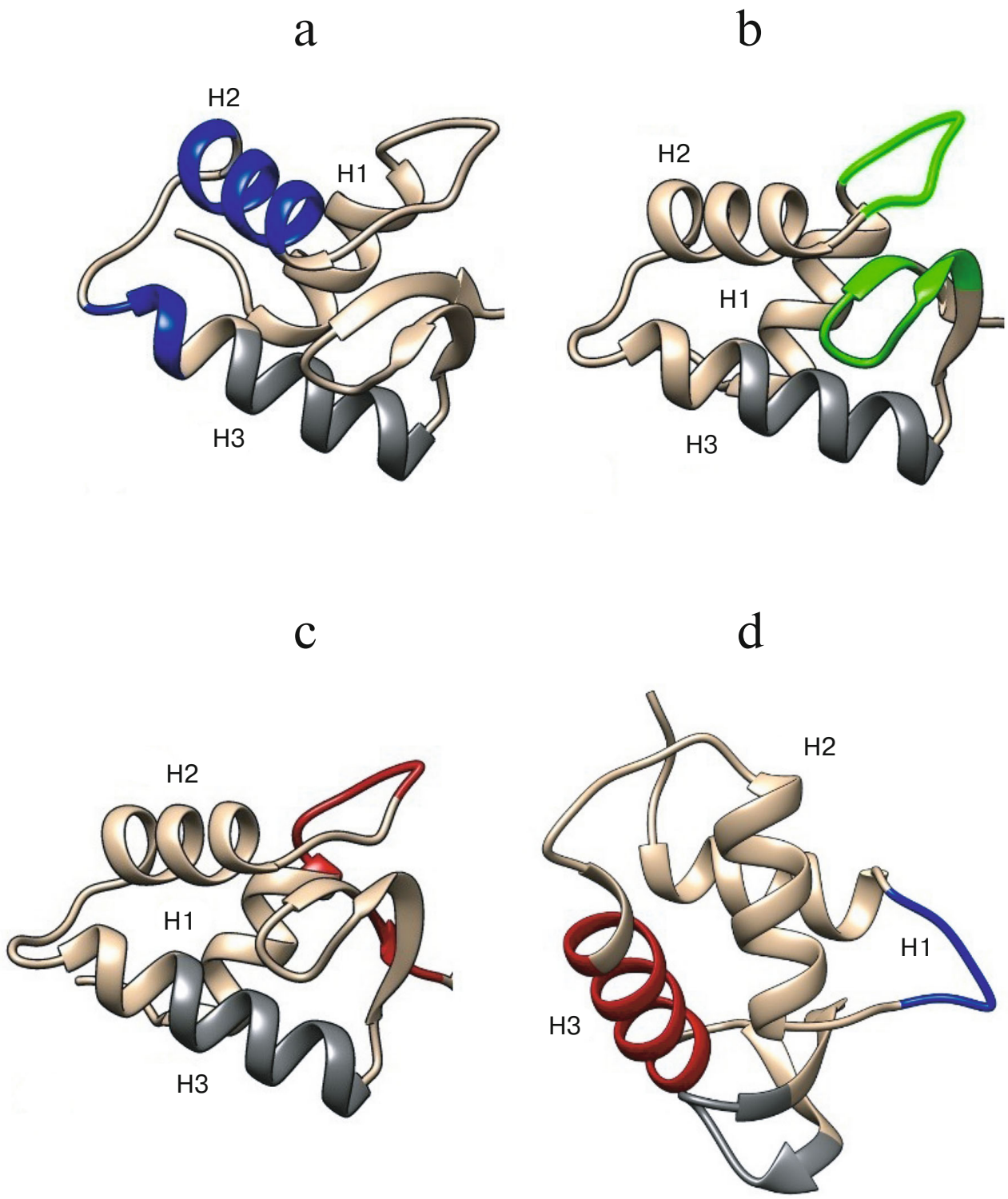

Fig. 3. H1 globule surfaces interacting with DNA of the nucleosome and linkers, according to data of various authors. a) According to Fan and Roberts [5], linker fragment is contacted by regions of helices H2 and H3 (shown in blue); b) according to data of Cui and Zhurkin [4], linker DNA contacts β-strand and loop H1-H2 (shown in green); c) a model built by Bai and coauthors [8], which predicts contacts of linker DNA with loop H1-H2 and a fragment of β-strand that belong to the C-terminal fragment (shown in red). Surface of H3 helix interacting with nucleosomal DNA is shown in gray in all the three models; d) according to X-ray analysis data obtained by Bai and coauthors [17], nucleosomal DNA is contacted by amino acids of β-strand (gray), while linkers contact with helix H3 (red) and a loop between helices H1 and H2 (blue).

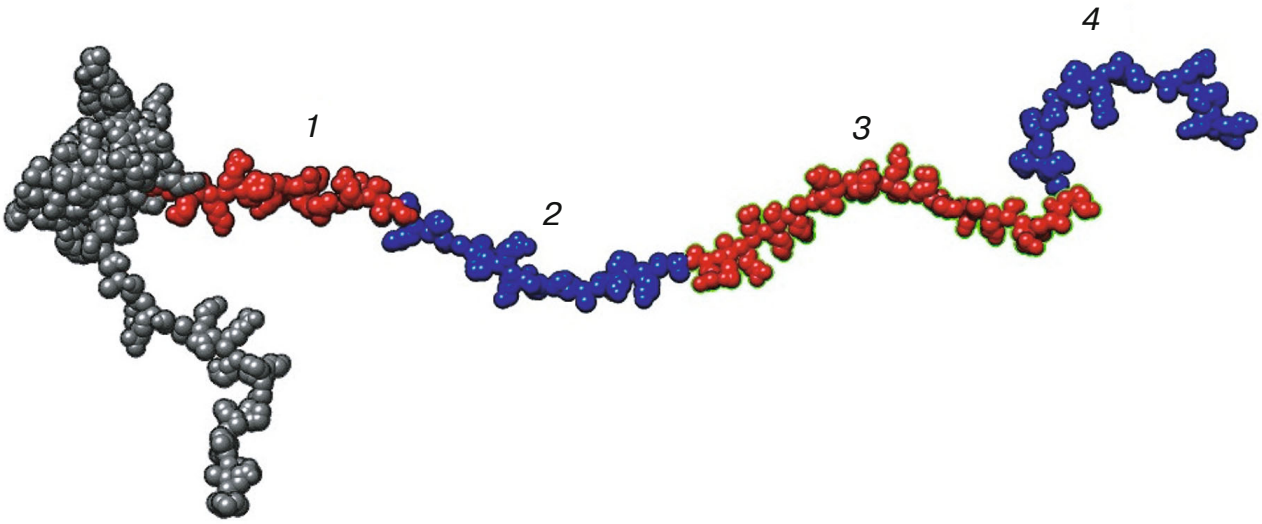

Fig. 4. Scheme of disposition of C-terminal portion fragments used in experiments. In one experiment, fragments 1 and 3 were swapped; in other experiments, amino acid residues of domain 3 were randomly changed.

nucleosome is contacted by different amino acid residues of the globule in different linker histone variants. This may determine the different affinities of these variants to the 30-nm fibril depending on its compaction level [16].

An alternative model of linker histone binding was proposed based on X-ray analysis of a complex of the nucleosome with the globular domain of chicken linker histone H5 (at 3.5 Å resolution) along with spin-label NMR [17]. According to this model, the H5 globule binds to the nucleosome without displacement relative to the symmetry axis. Thus, residues of the β-strand bind to nucleosomal DNA, helix H3 interacts with one linker, while the loop H1-H2 contacts the other one. The model does not contradict the experimental data obtained previously, but does not rule out an alternative way of linker histone binding, which is determined by, for instance, steric limitations in supernucleosomal structures. Therefore, according to cryoelectron microscopy data, being a part of the 12-nucleosome fibril, the H1 globule binds shifted relative to the symmetry axis of the nucleosome [18].

The N-terminal domain of linker histones is 20-35 a.a. in length. It can be divided into two distinct regions. The N-terminal region is enriched with alanine and proline as well as more hydrophobic amino acid residues, and it does not form stable complexes with DNA [19]. Second region has one arginine and five lysine residues, and its sequence is similar to that of N-terminal region of histone H3 [20]. It was demonstrated by means of high resolution NMR, IR spectroscopy, and circular dichroism that the N-terminal fragment is unstructured in aqueous solvent, while most of this region adopts α-helical conformation in trifluoroethanol. Binding of DNA to a linker histone may also induce formation of secondary structure within the N-terminal fragment [21, 22]. High positive charge of this fragment and its proximity to the globular domain may stabilize DNA binding to the globular domain of the linker histone [21].

The C-terminal domain of linker histone is approximately 100 a.a. long. It is the least conserved region among different histone variants [23]. This region is unstructured in solution and forms a stochastic random coil. Nevertheless, there are IR spectroscopy data indicating that upon DNA binding, the C-terminal domain acquires secondary structure [24, 25]. Binding to the nucleosome causes formation of a compact globule out of secondary structure elements [26]. This observation may explain why in some organisms, e.g. *Tetrahymena thermopila*, H1-like proteins do not have a defined globular domain [11]. The C-terminal fragment of linker histones is enriched with lysine, alanine, and proline, and it carries high total positive charge (~30-50). The highest positive charge in this region is typical for linker histone variants present in terminally differentiated cells (H1.0 and H5) [27]. It was shown that the ability of the C-terminal domain of histone H1.0 to change linker DNA structure and promote polynucleosome oligomerization is determined by two sequence fragments (Fig. 4, shown in red) that are located at short distance from each other [28]. At the same time, properties of amino acid residues of these fragments, their size, charge, and hydrophobicity, rather than a certain sequence play a key role in the linker histone binding to the nucleosome [29, 30]. So, no changes in ability of H1.0 to bind DNA and stabilize chromatin were observed after random rearrangements of amino acid residues within the first fragment (Fig. 4) [30]. Furthermore, fragments 2, 3, and 4 being placed instead of the first one, turned out to be functionally identical to

the latter. Thus, it is reasonable to assume that binding of the globular domain of linker histone to DNA is necessary for precise positioning of the C-terminal fragment, because the globular domain exclusively has affinity for the H1-binding site on the nucleosome. At the same time, the chromatin condensation function is performed by the C-terminus, which is apparently incapable of forming compact structure outside the binding site [30].

FUNCTIONAL FEATURES OF LINKER HISTONE VARIANTS

As mentioned above, most eukaryotes possess more than one variant of linker histones, which vary in amino acid composition (the globular domain has the most conserved sequence), length of polypeptide chain, and time and location of expression. The functional significance of such diversity is insufficiently studied. The fact that the number of different variants of linker histones correlates with complexity of the organism organization is very intriguing. So, in the fungus *Physarum polycephalum* [31] and the fruit fly *Drosophila melanogaster* [32], only one linker histone subtype was found, while in mammalian tissues 11 subtypes of histone H1 were found [23]. These 11 subtypes are divided into histones that are expressed in somatic cells during S phase of the cell cycle (H1.1-H1.5) and differentially expressed in somatic (H1.0 and H1x) and gametal cells and their precursors: H1t, H1T2, and HILS1 in testicular cells and H1oo in oocytes [23]. Variants of linker histones vary in time of expression in cells [33], exchange rate after binding to chromatin [34], and affinity to chromatin [35, 36]. For some H1 variants, predominant binding to eu- or heterochromatin [36] as well as differences in ability of different variants to condense chromatin [34] were demonstrated.

To elucidate the role of linker histone variants in development, their gene knockout was studied. Mice lacking the functional gene of histone H1.0 developed normally [37] as other linker histone variants are able to functionally substitute for this protein. No deviations were observed upon knockout of the *H1t* gene typical for testes [38], as well as upon knockout of *H1.1*, which is a main linker histone type [39]. No deviations were observed also in development of mice with double knockout of gene *H1.0* and one of the genes of murine homologs of human H1.2, H1.3, and H1.4 [40]. Deviations were only observed in case of triple knockdown of H1.2, H1.3, and H1.4 homologs, when embryos died during development. In this case, total histone H1 content was decreased by ~50% as compared to normal levels [41].

Dependence of the set of present linker histones on cell cycle phase was demonstrated in HeLa cells [10]. It was shown that during general decrease in content of mRNA and protein products of genes of all five somatic linker histones (H1.1-H1.5) upon butyrate-induced cell cycle arrest, the concentration of histone H1.5 and its mRNA dropped more markedly. This corresponds to data showing that levels of this histone are decreased in cells possessing weak or absent mitotic activity [42]. Cell cycle arrest at the G0/G1 phase caused first an increase, and then a decrease of histone H1.0 mRNA levels [10]. Increase in concentration of histones H1.2, H1.4, and H1.5 and of their mRNAs is typical for the S phase. Then an increase in expression of variants H1.0 and H1.3 occurred, which stopped by the end of the phase [10].

Different variants of histone H1 participate in gene regulation. Therefore, different linker histones are present on globin genes in erythroid cells and brain cells of chicken embryo in different quantities [43]. Using knockdown of genes of different linker histones in breast cancer cells, it was established that decrease in levels of various linker histones led to expression of different genes [44]. Decreased levels of linker histone H1.2 caused cell cycle arrest. Reduced levels of histone H1.4 caused death in some cell lines. Changed ratios of different linker histone content were also observed in induced pluripotent somatic cells, for which reduced synthesis of H1.0 and an increase in synthesis of H1.1, H1.3, and H1.5 was typical [45]. A pivotal role of the histone-like protein dBigH1 that differs from somatic H1 by an extended C-terminal fragment in regulation of gene expression was shown in *D. melanogaster* embryonic cells. In wild-type embryos, gene expression was not observed until cellularization. At the same time, in case of dBigH1 knockout, many RNA polymerase elongation complexes were found in embryonic chromatin at the same development stage, while embryos died in the middle of the developmental process [46]. Differences in expression of linker histone variants mostly correlated with changes in transcription and replicative activity of cells. These data suggest that the majority of functional differences between these variants are determined by different stability of binding to chromatin, which leads to different compaction levels and, in turn, to different functional activity of chromatin.

Affinity of linker histones to chromatin was a subject for several studies both *in vitro* and *in vivo*. In experiments on incorporation of human variants of linker histones to minichromosomes assembled using *D. melanogaster* embryonal extracts, it was established that human somatic linker histones can be classified as strong condensers (H1.0, H1.4, H1.5, and H1.x), intermediate condensers (H1.3), and weak condensers (H1.1 and H1.2) [47]. In another series of experiments, binding of H1 to long chromatin fragments and scaffold-attachment regions (SAR) was analyzed [35]. Using this method, highest affinity to chromatin was demonstrated for H1.3, H1.4, and H1.0, intermediate affinity for H1.2 and H1.5, while histone H1.1 had the lowest affinity to chromatin. It is worth mentioning that the affinity to chromatin does not always correlate with the condensation ability. This may be associated with different limitations determined by the

experimental technique, and by the fact that these two activities are attributed to different fragments of the H1 molecule.

To determine linker histone *in vivo* exchange rate, fluorescence recovery after photobleaching (FRAP) experiments were carried out in cells infected with herpes virus HSV-1 [48]. These studies revealed that histones H1.4 and H1.5 had lower exchange rate as compared to H1.0, H1.1, H1.2, and H1.3. Interesting, all the fast-exchanging histones (except for histone H1.3) possessed C-terminal regions that were shorter than those in slowly exchanged variants. FRAP experiments have also demonstrated dependence of H1 exchange rate on functional state of chromatin: the exchange rate was higher in transcriptionally active chromatin, as compared to inactive chromatin [49].

Affinity to chromatin and linker histone exchange rate strongly depends on certain experimental conditions used for evaluating these parameters. Particularly, experimental protocols *in vitro* do not take into account the influence of interaction with cell proteins on H1 exchange rate.

Despite the fact that stabilization of compact chromatin structure was always considered as the main role of linker histones, their interaction with other cellular proteins should be mentioned as well. The interaction of histone H1 with at least 16 proteins has been demonstrated [50]. Transcription factor YB-1, DNA-binding protein PURα, and an apoptotic endonuclease DFF40 are among these proteins [51, 52]. As for the latter, it was shown that specificity of its interaction with linker DNA is determined by interaction with the C-terminal fragment of linker histone H1 [53, 54]. It was also demonstrated that DFF40 could be activated by separate 48-a.a. fragments of the H1 C-terminal part.

Study of interactions of different fragments of histone H1.0 with nuclear proteins revealed that binding to cellular proteins is realized by means of not only the C-terminal, but also the globular domain. Furthermore, the latter provides ~75% of all interactions [55]. At the same time, precise location of binding sites for the majority of cellular proteins, as well as biological significance of the observed interactions, remain unknown. Analysis of known interactions between H1-binding proteins allows dividing them into three groups: proteins responsible for synthesis and processing of rRNA, spliceosomal proteins, and ribosomal proteins. The presence of the majority of these proteins in the nucleolus suggests the biological significance of the revealed interactions [55].

ROLE OF LINKER HISTONES IN FORMATION OF 30-nm FIBRIL

Regarding linker histones, one of the most important questions is determination of their precise location within the chromatosome and the 30-nm fibril [56]. There are not many data regarding the 30-nm chromatin fibril. These data are also so contradictory that some authors call into question biological significance of this structure [57]. So, according to recent data obtained using a high-scale chromatin conformation capture (Hi-C) method and electron microscopy combined with tomography, the 30-nm fibril may be absent from, at least, nuclei of transcriptionally and mitotically active cells [58, 59]. However, modern techniques revealed the classical 30-nm fibril in nuclei of quiescent cells such as starfish spermatozoa [58]. These fibrils were also found in the polytene chromosomes in insects [60]. Chromatin fragments isolated from cell nuclei and assembled on artificial templates with positioning sequences fold into a similar structure [61, 62].

While considering the question of existence of this level of DNA compaction in the interphase nucleus, it should be kept in mind that the 30-nm fibril is very labile: passing of just one RNA polymerase complex is sufficient for its destruction [60, 62]. The fibril structure is easily distorted [62]. This may also partially explain difficulties in finding the fibrils in interphase nuclei.

Earlier studies using electron microscopy [63] suggested two 30-nm fibril models: one-start and two-start helices. The one-start DNA model suggests that nucleosomes stack up against one another and form a uniform helix; linker DNA is assumed to be folded inside the helix [64]. The two-start double superhelix suggests that nucleosomes stack up against one another in a manner generating two stack helices; linker DNA fragments are situated between the two nucleosomal stacks [65].

Structures resembling two-start helix were observed using electron microscopy and tomography in nuclei of cells with inactive chromatin, such as echinoderm spermatozoa or avian erythrocytes [66]. Observed topological variations in these structures, in the authors' opinion, is best explained with the twisted ribbon model – a two-start helix model prototype, which differs from the latter by lower compactness and lack of interaction between histones of lateral surfaces of nucleosomes. They considered linker DNA fragment length as a key factor affecting the fibril structure.

The X-ray crystal structure of a tetranucleosome with 20-21-bp linker internucleosomal DNA fragments determined at 9 Å resolution supports the two-start model [67]. The structure represents a two-start helix in which a nucleosome is 70° rotated relative to the preceding one. There are reasonable doubts regarding biological significance of this structure as the tetranucleosome was assembled without linker histones. Moreover, small length of linker fragments and their strict conformational limits do not allow incorporation of the linker histone globule to its binding site [68]. It should be mentioned also that such chromatin folding into the fibril rules out interaction between nucleosomes that was observed in experiments

on chemical crosslinking of chromatin fibril proteins in solution [69]. The presence of histone H1 did not change the pattern of these crosslinks.

More convincing evidence in favor of two-start helix was obtained with high resolution cryoelectron microscopy of artificial polynucleosomes containing 12 nucleosomal repeats 177- and 187-bp in length that were assembled in the presence of linker histone [18]. High resolution of 11 Å achieved by the authors allowed unambiguous tracing the DNA molecule passage: it corresponded to the two-start helix. Nucleosomes in this structure are organized into three tetramers on a "head-to-head" principle. Interaction between such tetramers is apparently stabilized predominantly by asymmetrically bound H1, whose globules face toward the interacting tetramer surfaces.

Recent calculations made for two-start helical models with various linker lengths that revealed several possible stable configurations of this fibril also favor the two-start helix model [70]. These studies revealed, in particular, that right-handed helix is less energetically favored as compared to left-handed helix. Results of recent ultracentrifugation and electron microscopy studies of model polynucleosomes also support the two-start model [61]. It is significant that compaction order was the same for fibrils with both regular and irregular linker lengths. However, changing the length of the linker fragment so that the number of nucleotides does not coincide with the formula 10n + 5 caused strong chromatin decompaction. Note that it was precisely the 10n + 5 ratio that was observed *in vivo*; quantitative techniques also suggest preferability of linker lengths satisfying the 10n or 10n + 5 rule [62, 70].

The one-start model is currently based on data of electron microscopy of polynucleosomes with various nucleosome repeat lengths (177-237 bp, of which 146 bp are accounted for the nucleosome-positioning DNA sequence) assembled at low concentration of divalent cations (1.6 mM $MgCl_2$) and containing one linker histone per nucleosome [71, 72]. Depending on the nucleosome repeat length, fibrils belonged to two classes were obtained, which varied in diameter and number of nucleosomes per unit length. Linear increase of fibril diameter that one might expect in case of the two-start organization of fibril of this kind was not observed. It is worth mentioning that only one-start or multiple-start polymorphic helix may correspond to these data [62, 68].

One has to take into account that results of mathematical modeling of chromatin fibril suggest possible existence of one topology in addition to the two above-mentioned ones – multiple-start polymorphic helix, whose properties should (according to calculations) strongly depend on the linker fragment length [15].

In summary, one can state that the previous prevalent conception of the 30-nm fibril as a strongly ordered structure that is an obligatory level of interphase chromatin compaction does not correspond to the available data. There is also no reason to assume that such a fibril is stable, as all the proposed models of its structure carry incompletely compensated negative charge. Indeed, one RNA polymerase complex turns out to be sufficient for destruction of the 30-nm fibril [60]. Hence, the 30-nm fibril possesses a polymorphic and labile structure depending on a number of factors such as linker DNA fragment length and participation of proteins that interact with DNA and chromatin [62]. Any fibril may have a number of conformations, which are probably notably different.

The role of histone H1 in maintaining structure of the 30-nm fibril apparently consists, first, in stabilization of the compact state of chromatin [73]. Therefore, sedimentation coefficient of fibrils assembled in the presence of linker histone is higher compared to that of chromatin assembled without it, indicating their higher compactness [61]. Cryoelectron microscopy data also suggest an important role of linker histone in maintaining the 30-nm fibril structure: its globules provide interaction between tetramers of nucleosomes folded in "head-to-head" manner [18].

Among the structural features of linker histones allowing them to perform this function, first, an extended C-terminal domain carrying a significant total positive charge should be mentioned. Upon binding to DNA in the linker region, this domain neutralizes negative charge of sugar-phosphate DNA backbone, thus preventing Coulomb repulsion between two like-charged DNA chains that approach each other during 30-nm fibril formation [68]. Different linker histone variants of higher eukaryotes have different C-terminal domains that vary in length and amino acid composition [23]. These variations may affect structure of the 30-nm fibril that contains a variant of this histone [18, 72].

The second important for the 30-nm fibril compaction structural element of linker histone is its globular domain binding to DNA close to its exit from the nucleosome and, thus, positioning the C- and N-termini of histone, as well as preventing spontaneous partial DNA bend out of histone octamer (the so-called nucleosome "breathing") [74]. In one of the proposed fold models, globular domains of linker histones face each other, which apparently provides additional stabilization of the fibril [18]. According to one of these hypotheses, the order of H1 globular domain binding determines geometry of the 30-nm fibril [17]. It is also appropriate to assume that regulation of histone H1 binding and, as a result, chromatin compaction may be realized in a cell by means of posttranslational modifications of this protein, changing electrostatic properties of its C-terminus.

The data described above indicate important functions of linker histone. This protein was acquired by eukaryotes early in the course of their evolution and, based on the results of comparative analysis, independ-

ently of the nucleosome core histones. The structure of the globular domain was determined with both X-ray analysis and NMR. However, we still do not have a clear view of histone H1 interaction with nucleosomes and other proteins.

It is a well-established fact that various histone H1 variants differ in affinity to chromatin; there is evidence that these differences are functionally significant. At the same time, it was proved that in the absence of one histone, other histones are able to substitute for it without notable physiological deviations.

The best-studied function of linker histone is stabilization of the 30-nm chromatin fibril. Thus, differential regulation of structural and functional states of different regions of chromatin may be achieved by regulation of binding of different H1 variants. It is well known that these functions are performed via H1 binding to nucleosomes close to the symmetry axis (Fig. 1). However, there is still no unambiguous model of interaction of H1 (as well as of its globular domain) with the nucleosome. The reason for that is, first, lack of a precise atomic-level model of the predicted site for binding of this protein in view of limited data regarding relative topology of linker DNA in the 30-nm fibril, which also complicates studying with quantitative techniques.

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