Chromatin structural transitions in *Drosophila* embryo cell-free extract result in a high conformational flexibility of nucleosomal DNA

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Abstract DNA within chromatin has considerably more restricted flexibility in comparison with naked DNA. This raises the main question of how the functioning multi-enzyme complexes overcome the nucleosomal level of DNA packaging. We studied the DNA conformational flexibility of reconstituted chromatin in a cell-free system derived from *Drosophila* embryo extracts. Using this system, we have found evidence for a energy-independent chromatin remodelling process that efficiently destabilizes the nucleosome structure resulting in a high conformational flexibility of nucleosomal DNA. The described chromatin remodelling process may lay on the basis of defined molecular principles governing the molecular heterogeneity of chromatin structures in vivo.

Key words: Chromatin; Nucleosome; DNA topology; Nucleosome remodelling

1. Introduction

DNA in the cell nuclei is packed into histone globules, nucleosomes, and thereafter, into compact nucleosome fibers. This raises the main question of how the functioning multi-enzyme complexes overcome the nucleosomal level of DNA packaging. Opinions in the literature differ widely on this question. There are assumptions of complete conservation of the nucleosomal structure, as well as an assumption of considerable changes at the nucleosomal level of organization, up to complete nucleosome dissociations from DNA. Recently, a growing number of supportive evidence was found for the hypothesis that the conformational changes take place within the nucleosome, while the nucleosomal structure, as such, is preserved [1–3]. In this connection, the model representing a transcriptionally active nucleosome as a structure with the features of nucleosomal organization, within which DNA preserves some properties of free DNA, appears to be very attractive.

DNA within chromatin has a considerably more restricted flexibility in comparison with the naked DNA. Free DNA in solution is capable of changing its helical twist in response to temperature and other environmental stimuli [4–6]. In circular DNA, in the presence of topoisomerase, these conformational changes are attended by the alterations in the DNA linking number, that can be assayed by agarose gel electrophoresis. As expected, bacterial DNA behaves in a similar way [6–8]. Quite another picture is observed for eukaryotic DNA. The nuclear DNA is supercoiled and stabilized by interacting with histone octamers in nucleosomes [4,5]. The linking number of intact SV-40 minichromosomes [7,9,10] remains unchanged in response to variations in the temperature. Considerable attempts to reconstitute a flexible nucleosome structure using either normal or modified (trypsinized or hyperacetylated) mammalian histones were not successful [8,11–13].

However, some endogenous transcriptionally active yeast minichromosomes respond in vivo to a temperature shift by changes in the linking number [14,15]. This peculiarity of the yeast chromatin was previously attributed to the differences in primary structure between yeast histones and histones of higher eukaryotes. It was shown that yeast histones H3 contain alanine instead of the cysteine 110 [5,16,17]. Therefore, yeast nucleosomes may be incapable of stabilization by intranucleosomal histone H3 dimerization as opposed to the nucleosomes of higher eukaryotes [5,17].

However, later, it was found that the transcriptionally active mammalian minichromosomes can also change their linking number with the temperature [18]. At the same time, it was shown that DNA in the bulk yeast nucleosomes and in the fraction of 'unfolded' mammalian nucleosomes with a splitted histone H3-H3 disulfide bond [19–22] was not more flexible than DNA of bulk mammalian nucleosomes [4,23]. The results of these experiments provided evidence to re-examine the conception that high the conformational flexibility of nucleosomal DNA may be automatically created through modifications of the histone primary sequence [5,10,24]. Some additional changes must be introduced in the nucleosome core to create a dynamic, transcriptionally potent chromatin structure. These transitions may be a result of virtual chromatin remodelling processes occurring in active gene domains.

We studied the DNA conformational flexibility of reconstituted chromatin in a cell-free system derived from *Drosophila* embryo extracts [25]. Using this system, we have found evidence for a energy- independent chromatin remodelling process that efficiently destabilizes the nucleosome structure resulting in a high conformational flexibility of nucleosomal DNA.

2. Materials and methods

Mammalian core histones were isolated as described previously [26] and were analyzed on 13% SDS polyacrylamide gels [27].

Chromatin reconstitution extracts [25] from 3–6 h old *Drosophila* embryos were depleted of endogenous histones by incubation with 50 μg immobilized DNA per 500 μl extract for 30 min at 4°C with shaking [28,29]. A standard reconstitution reaction [30] contained 20 μl of histone-depleted extract, 100 μl extraction buffer EX (10 mM HEPES-KOH, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10% glycerol (v/v), 1 mM DTT, 0.2 mM PMSF), 80 mM KCl, 13.3 μl of 10× energy regeneration buffer McNAP (30 mM ATP, 300 mM creatine phosphate (Sigma), 30 mM $MgCl_2$, 10 ng/μl creatine phosphokinase (Sigma), 10 mM DTT), 650 ng plasmid DNA and purified core histones at a (1.5–2.5):1 (w/w) histone/DNA ratio in

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a final volume of 133 μl. Chromatin reconstitution was carried out for 6 h at 26°C. After reconstitution, chromatin was additionally purified over a spin-column containing 10 volumes of Sephacryl S-300 pre-equilibrated with EX buffer containing 80 mM KCl.

To induce chromatin remodelling, *Drosophila* extract was depleted of endogenous histones using immobilized DNA (see above), dialyzed in microcollodion bags (Sartorius, 10 000 MW limit) against EX buffer containing 80 mM KCl for 2 h at 4°C. In some experiments, extract was pre-heated at 75°C for 5 min. Remodelling was performed by incubation of 30–50 μl of purified chromatin (corresponds to 100–200 μg DNA) in the presence of 0.3–0.6 μl of dialyzed histone-depleted extract for 30 min at 26°C.

Digestion with micrococcal nuclease was performed as described previously [28,30], scaled to the volume of the chromatin sample.

To analyze histones in reconstituted chromatin, chromatin assembled on dynabeads-bound DNA was washed with 1 ml EX-80 buffer and then extracted with 0.5 ml 2 M NaCl for 1 h at 0°C. Histones in the supernatant were precipitated with 25% TCA, dissolved in 0.5 M HCl and finally precipitated with six volumes of acetone and analyzed on 13% SDS polyacrylamide gels.

For analysis of temperature-dependent DNA flexibility, 20 μl of chromatin solution was incubated for 40–60 min at 35°C or 2–4 h at 5°C with 0.5 U of *Drosophila* topoisomerase I (Pharmacia). Reactions were stopped with 10 μl 2.5% (w/v) sarkosyl/100 mM EDTA. DNA was isolated and analyzed on 1.2% agarose gels containing 3–5 μM chloroquin (Sigma).

3. Results

Chromatin was assembled using a circular 6250 bp plasmid DNA (pXX3.2 [31]) and histones isolated from CV1 cells (green monkey kidney cell line). Reconstituted chromatin was purified over a 'spin-column' containing 10 volumes of Sephacryl S-300. To induce nucleosome remodelling, chromatin was treated with a small amount of dialyzed histone-depleted *Drosophila* embryo extract.

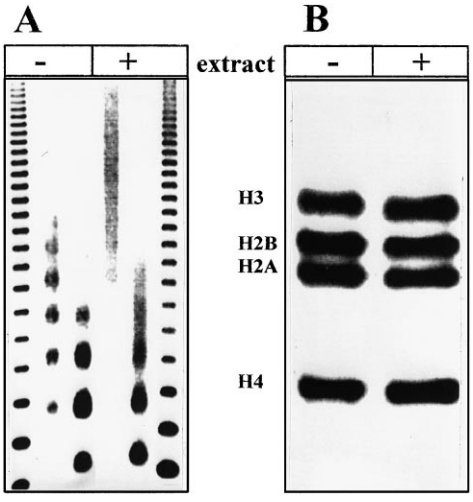

Fig. 1. Structural analysis of chromatin remodelled in *Drosophila* embryo cell-free extract. (A) Analysis of nucleosome arrays with micrococcal nuclease. Chromatin was reconstituted in a standard reaction and purified over a 'spin-column' containing 8–10 volumes of Sephacryl S-300. Then, dialyzed histone-depleted *Drosophila* extract was added and the reaction was incubated at 26°C for 30 min, then, chromatin samples were digested with micrococcal nuclease for 1 and 5 min. The marker DNA is a 123 bp DNA 'ladder' (Boehringer Mannheim). The gel is shown as a negative image. (B) Analysis of core histones of chromatin remodelled in *Drosophila* extract. Chromatin was assembled on on dynabeads-bound DNA. Chromatin was washed twice in EX-80 buffer and incubated in the presence of embryo extract. Histones were re-extracted and analyzed on 13% SDS polyacrylamide gels. Positions indicating migration of individual histones are shown at the left.

To assess the integrity of reconstituted chromatin, we assayed nucleosome arrays by micrococcal nuclease digestion (Fig. 1A). DNA within the nucleosome core is highly protected by histones to micrococcal nuclease cleavage and a partial digestion with micrococcal nuclease produces a typical 'ladder' of DNA fragments corresponding to multimers of nucleosome-sized fragments. Digestion of a control sample of reconstituted chromatin resulted in a set of well-resolved oligonucleosomal fragments (Fig. 1A). However, after treatment with *Drosophila* embryo extract, chromatin became more resistant to micrococcal nuclease cleavage producing a noticeably less-pronounced nucleosomal 'ladder' (Fig. 2B). It is important to note that incubation of chromatin in *Drosophila* embryo extract was not accompanied by any noticeable effect on the integrity of histones in reconstituted chromatin (Fig. 1B). Therefore, these chromatin structural transitions did not result from non-specific degradation of histones in assembled nucleosomes.

To test the temperature-dependent DNA flexibility, chromatin samples were splitted in two portions and relaxed with *Drosophila* topoisomerase I either at 5°C for 2–3 h or at 35°C for 30–40 min (Fig. 2A). Protein-free DNA unwinds by 0.011°/bp/°C ([6] and references therein). The overall unwinding of 6.2 kb of naked DNA (the length of our plasmid DNA) for a temperature shift of 30°C is predicted to produce about 5.7 additional superhelical turns, that fits well with the experimental data (data not shown). In control chromatin, the DNA of the 35°C sample was unwound by 1.5–2.0 turns relative to that of the 5°C sample. This amount of unwinding of 20–30% roughly corresponds to that of internucleosomal linker DNA free of histone octamers. When the extract was added, the DNA conformational flexibility was strikingly increased, that was accompanied by an increase of total DNA supercoiling (Fig. 2A). Relaxation of extract-treated chromatin at 35°C resulted in accumulation of 4–5 additional supercoils when compared to relaxation at 5°C. This amount of temperature-mediated DNA unwinding is about 70–90% of the value observed for naked DNA in the same temperature range. This means that the re-modelled nucleosome restrains only 1/4–1/6 of the DNA restrained by a normal nucleosome. Such a high share of unrestrained DNA arising in chromatin after incubation with extract must be due to altered DNA-histone interactions in the DNA that are usually restrained by nucleosomes. This includes DNA associated with the histone octamer (146–160 bp) as well as part of the adjacent linker DNA [11,12]. A similarly high degree of rotational flexibility has been observed in vivo in yeast minichromosomes [14,15] and transcriptionally active BPV-based minichromosomes in intact mouse cells and in isolated nuclei [18], which may represent structural features of transcriptionally active chromatin (for a discussion see [4]).

When the *Drosophila* extract was pre-heated at 75°C for 5 min before addition to chromatin, the effect on the temperature-mediated DNA flexibility was completely abolished (data not shown). Short treatment of extract at a high temperature results in denaturation and precipitation of most macromolecules leaving RNA and nucleoplasmin in solution. The molecular chaperone nucleoplasmin [32], a histone-binding protein with an enhanced specificity for H2A-H2B pairs, was previously shown to have an important role in the tran-

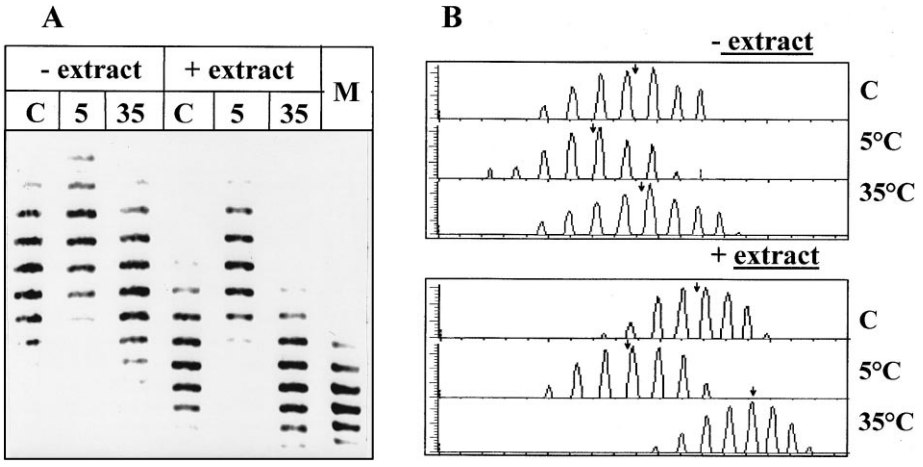

Fig. 2. Analysis of the DNA conformational flexibility in *Drosophila* embryo cell-free extract. Chromatin was reconstituted in a standard reconstitution reaction and purified over a 'spin-column' containing 8–10 volumes of Sephacryl S-300. Then, topoisomerase I and dialyzed histone-depleted extract were added and the reaction was incubated at 26°C for 30 min. Chromatin was divided in three samples, one sample was used for immediate DNA isolation (lane: 'C'), two other samples were additionally relaxed at 5°C (lane: '5') and 35°C (lane: '35') for 2–3 h and 40 min. Lane 'M' is 6250 bp naked supercoiled plasmid isolated from a bacteria source, the same plasmid was used for chromatin reconstitutions. The supercoiling level of marker plasmid corresponds to supercoiling introduced by 30–31 assembled nucleosomes. The maximal possible amount of assembled nucleosomes at a current nucleosome spacing (185 bp, see Fig. 1A) is 34 nucleosomes. The gel is shown as a negative image. (B) Densitometric analysis of the gel shown in A.

scription factor-induced nucleosome displacement [33,34], selective removal of linker histones from somatic nuclei [35] and decondenzation of sperm chromatin [36,37]. Our results indicate, however, that in our case, nucleoplasmin is not involved in the chromatin remodelling we describe.

It is worth to note that some of the described above chromatin remodelling effects may be observed in a crude chromatin reconstitution mixture, if the mixture will be depleted of ATP after completion of chromatin assembly (data not shown). This implies that in *Drosophila* cell-free extract, a dynamic equilibrium may occur between ATP-dependent chromatin assembly processes [25,38–41] and the described energy-independent chromatin remodelling.

4. Discussion

We found evidence for a nucleosome remodelling process occurring in *Drosophila* embryo cell-free extract. This process results in transition of a normal nucleosome to the structure still retaining basic features of a nucleosomal particle such as a discrete micrococcal nuclease digestion pattern and a full complement of core histones (Fig. 1). At the same time, the resulting structure exhibits high levels of DNA conformational flexibility (up to 90% of this value for naked DNA). This remodelling process also results in an increase of total DNA supercoiling, introduced by the nucleosome. In contrast to other described chromatin remodelling processes [40–45], the remodelling we describe does not require hydrolysis of ATP as an energy source and is spread over a continuous nucleosome array rather then being restricted to single nucleosomes bound to specific transcriptional activators.

One nucleosome particle introduces one supercoil in circular closed DNA, however, DNA is making 1.75 turns around a histone octamer [46]. This paradox can be explained by the local alteration in the DNA helical pitch on the surface of the nucleosome [8,46]. It is postulated that nucleosomal DNA has an overall DNA double helix pitch of 10.0 bp instead of a 10.4 bp pitch for free DNA in solution. Loosening of DNA-histone interactions must result in partial restoration of the DNA double helix pitch to the values more close to those of naked DNA in solution. This must result in an increase of total nucleosome supercoiling, since 1.75 DNA supercoils around the core particles will be less counter-balanced by overtwisting of DNA on the nucleosome surface. Therefore, the increase of DNA supercoiling that was observed upon nucleosome remodelling may reflect the local disruptions of histone-DNA contacts. This is consistent with the high degree of DNA conformational flexibility in remodelled nucleosomes. Restricted temperature-dependent dynamics of nucleosome DNA are currently explained by extrusion of solvent molecules (water) from the interaction with the DNA phosphate backbone that occurs due to a tight interaction of the DNA backbone with the histone octamer [6,47,48], i.e. the interaction of the DNA phosphates with H_2O molecules are replaced by histone-DNA interactions. Therefore, destabilization of DNA-histone interactions should result in an increased conformational flexibility of DNA.

A more 'open' nucleosome structure may explain the less-pronounced micrococcal nuclease digestion pattern (nucleosomal 'ladder') of remodelled chromatin (Fig. 1A). Micrococcal nuclease cleaves DNA mainly at linker DNA between nucleosomes and more diffused remodelled nucleosomes may protect linker DNA from digestion. This is consistent with the observation that the remodelled chromatin is more resistant to micrococcal nuclease digestion (Fig. 1A).

Considering that an increased conformational flexibility of DNA (up to 60–70% of this value for naked DNA) was found in vivo only for transcriptionally active chromatin [14,15,18], there are good grounds to argue that a high flexibility of DNA may represent one of the features that distinguishes the transcriptionally active 'open' chromatin fraction from the bulk chromatin (reviewed in [4,23]). A less-pronounced micrococcal nuclease digestion pattern was also widely reported as a hallmark of transcriptionally active chromatin (for review see [46]). Thus, the described energy-independent chromatin remodelling process may lay at the basis of defined

molecular principles governing the molecular heterogeneity of chromatin structures in vivo.

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