

Function of nucleophosmin/B23, a nucleolar acidic protein, as a histone chaperone

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Abstract We previously identified and purified a nucleolar phosphoprotein, nucleophosmin/B23, as a stimulatory factor for replication from the adenovirus chromatin. We show here that nucleophosmin/B23 functions as a histone chaperone protein such as nucleoplasmin, TAF-I, and NAP-I. Nucleophosmin/B23 was shown to bind to histones, preferentially to histone H3, to mediate formation of nucleosome, and to decondense sperm chromatin. These activities of B23 were dependent on its acidic regions as other histone chaperones, suggesting that B23/nucleophosmin is a member of histone chaperone proteins. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Acidic chaperone; Chromatin; Decondensation; Nucleolus; Nucleosome assembly

1. Introduction

The change of nucleosome structure is a key determinant of initiation of transcription and genome replication. After transcription and replication, remodeled and newly synthesized DNA are re-packed into nucleosome by nucleosome assembly proteins. Replication-coupled chromatin assembly factors, such as CAF-I [1] and RCAF complex [2], have been identified from several organisms. These factors bind to newly synthesized histones and deposit them to DNA in a DNA replication-dependent manner. In addition to these replication-dependent histone deposition factors, histone binding proteins such as nucleoplasmin, N1/N2, NAP-I, and Spt6 also have shown to play roles in nucleosome assembly independent of DNA replication *in vitro* [3]. These proteins bind to core histones and transfer them to naked DNA. In this regard, these proteins are categorized into 'histone chaperones'. In addition to histone chaperones, spacing factors, such as ACF [4], are suggested to be required to form physiological nucleosome arrays.

We have purified and characterized cellular acidic proteins, termed template activating factors (TAF), using the adenovirus chromatin DNA replication system as an assay system [5,6]. Since adenovirus DNA is complexed with viral basic core proteins forming a chromatin-like structure, a remodeling factor(s) of the viral chromatin structure is needed prior to initiation of transcription and replication. TAF-I was first

identified and purified from HeLa cells as a stimulatory factor for DNA replication from the viral chromatin [5]. TAF-I remodels not only the viral chromatin but also the cellular-type chromatin by interacting with nucleosomal histones [7]. Furthermore, TAF-I is associated with free histones and transfers them to naked DNA to form the nucleosome structure [6]. In addition, TAF-I decondenses *Xenopus* sperm chromatin [8] as do nucleoplasmin, NAP-I, and DF31. From these biochemical properties, TAF-I, which was originally identified as a stimulatory factor of the replication from adenovirus chromatin, is categorized into the family of histone chaperones. Since TAF-I and TAF-II, the latter of which was shown to be identical to NAP-I [9], contain functionally important acidic regions, we proposed the term 'acidic chaperone' for these proteins [3]. Recently, using the DNA replication system from the adenovirus chromatin, we have purified a novel factor consisting of two splicing variants of nucleolar phosphoprotein, nucleophosmin/B23 [10]. Two subtypes of nucleophosmin/B23 termed B23.1 and B23.2 differ from each other in short specific regions at their carboxyl-termini. Nucleophosmin/B23 is localized mainly in nucleoli and suggested to be a factor for ribosome biogenesis because of its ability to digest RNA [11] and to bind to nucleic acids [12]. However, a bona fide cellular function(s) of B23 in nucleoli has not yet been clarified.

Here, we show that B23 proteins function as histone chaperone proteins using well-characterized assays including histone binding, nucleosome assembly, and sperm chromatin decondensation assays.

2. Materials and methods

2.1. Preparation of proteins

Core histones, histone H2A/H2B, and histone H3/H4 were purified from HeLa cells as described previously [13].

Histidine-tagged recombinant human B23 proteins (His-tagged rhB23) and glutathione *S*-transferase (GST)-tagged yeast NAP-I were generated by *Escherichia coli* and purified as described [10].

cDNA encoding hB23.1 was cloned in frame into the *Bam*HI site of pGEX2T, to generate GST-fused hB23.1. *E. coli* BL21 cells were transformed by pGEX2T-hB23.1, and cultured until OD₆₀₀ reached 0.4–0.6. Expression of GST-hB23.1 was induced by adding isopropyl β-D-thiogalactopyranoside. Cells were suspended in buffer A (50 mM Tris-HCl pH 7.9, 1 mM MgCl₂, 0.1 mM PMSF, and 0.1% Nonidet P-40) containing 150 mM NaCl, and disrupted by extensive sonication, and cell extracts were recovered by centrifugation. GST-hB23.1 was purified according to the manufacturer's instruction using glutathione-Sepharose CL4B (Amersham Pharmacia, Piscataway, NY, USA). GST-hB23.1 was further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by denature-renature protocol [14].

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For far-Western blotting, cDNA encoding hB23.1 was subcloned into *Nde*I- and *Bam*HI-digested pET14bk to generate rhB23.1. rhB23.1 was expressed in *E. coli*, and purified as described [10].

2.2. Glycerol density gradient assay

Core histones (5 μ g) were incubated with or without rhB23 proteins (5 μ g) at 30°C for 30 min in buffer H (20 mM HEPES–NaOH pH 7.9, 0.5 mM EDTA, 10% (v/v) glycerol, 1 mM DTT, and 0.1 mM PMSF) containing 150 mM NaCl, and then loaded onto 15–35% glycerol in buffer H containing 150 mM NaCl. Samples were centrifuged at 4°C for 18 h at 54 000 rpm in a TLS55 rotor (Beckman, Fullerton, CA, USA), and fractions (100 μ l) were collected from the top. Proteins in each fraction were separated by a 13% SDS–PAGE and were visualized by silver staining.

2.3. GST pull-down assay

GST or GST-hB23.1 (1 μ g) was mixed with 1 μ g of either core histones, histone H2A/H2B complexes, or histone H3/H4 complexes in buffer A containing 150 mM NaCl and stored on ice for 30 min. The mixture was incubated at 4°C for 2 h with 20 μ l of a glutathione–Sepharose CL4B bead suspension (Amersham Pharmacia). After the beads were washed extensively with buffer A containing NaCl, concentration of which is indicated in the figure legend of Fig. 1B, proteins were eluted from the beads by incubation with a SDS sample buffer, separated by SDS–PAGE, and visualized by silver staining.

2.4. Far-Western blotting

Far-Western blotting was performed as described previously [7]. rhB23.1 or core histones were phosphorylated by protein kinase A (PKA, Sigma, St. Louis, MO, USA) in the presence of [γ -³²P]ATP and used as probes.

2.5. Nucleosome assembly assay

Supercoiled plasmid DNA (pCHA [15], 4.9 kb) was relaxed by incubation with topoisomerase I (Takara Shuzo, Kyoto, Japan) at 37°C for 30 min. The supercoiling assay was performed essentially as described [16]. MNase digestion assay was carried out as described in the legend of Fig. 3B.

2.6. Sperm chromatin decondensation

The sperm chromatin decondensation assay was performed as described [8].

3. Results and discussion

Nucleophosmin/B23 proteins, consisting of two subtypes, i.e. B23.1 and B23.2, stimulate replication from the adenovirus chromatin, and their acidic regions are critical for the stimulatory activity. Here, we have examined whether B23 proteins function as histone chaperones. First, the histone binding activity of B23 proteins was examined by glycerol gradient sedimentation assays (Fig. 1). His-tagged rhB23 proteins were mixed with core histones purified from HeLa cells, and then subjected to centrifugation through a 15–35% glycerol gradient. Core histones alone were recovered in the upper fractions (Fig. 1A, upper panel), while those preincubated with rhB23.1 (middle panel) or rhB23.2 (bottom panel) were fractionated in the higher density fractions, indicating that both subtypes of hB23 proteins bind to core histones. Core histones consist of two H2A/H2B dimers and a H3/H4 tetramer. TAF-I and NAP-I preferentially bind to H3/H4 complexes and H2A/H2B complexes, respectively. To clarify the binding specificity of hB23 proteins in terms of histone subtypes, we carried out GST pull-down assays (Fig. 1B). GST-tagged rhB23.1 binds to all histone subtypes under less stringent conditions (Fig. 1B, lanes 9–11), while GST does not bind to any histone subtypes (Fig. 1B, lanes 6–8). GST-tagged rhB23.1 binds to histone H2A/H2B complexes less efficiently than to histone H3/H4 complexes in the presence of 150 mM

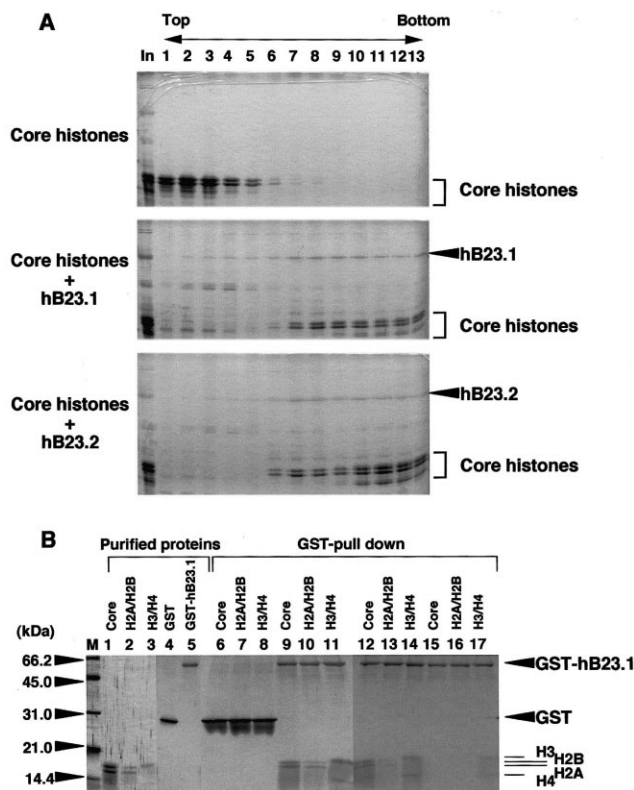


Fig. 1. B23 binds to core histones. A: Glycerol density gradient assay. Core histones (5 μ g) purified from HeLa cells, and rhB23.1 or rhB23.2 (5 μ g) were incubated and then subjected to the glycerol gradient sedimentation assay. Fractions were collected from the top and analyzed by 15% SDS–PAGE. Upper panel, core histones alone; middle panel, core histones and rhB23.1; bottom panel, core histones and rhB23.2. Lane 'In' indicates input. B: GST pull-down assay. GST (lanes 6–8) and GST-hB23.1 (lanes 9–17) were incubated with core histones (lanes 6, 9, 12, and 15), histone H2A/H2B complexes (lanes 7, 10, 13, and 16), or H3/H4 complexes (lanes 8, 11, 14, and 17). The mixtures were subjected to the GST pull-down assay. Proteins bound to glutathione–Sepharose beads after washing extensively with buffer A containing 150 mM (lanes 6–11), 250 mM (lanes 12–14), and 500 mM (lanes 15–17) NaCl were analyzed by a 13% SDS–PAGE and visualized by silver staining. Purified core histones (100 ng), histone H2A/H2B (100 ng), H3/H4 (100 ng), GST (500 ng), and GST-hB23.1 (500 ng) are shown in lanes 1–5. Positions of GST, GST-hB23.1, and histones H2A, H2B, H3, and H4 are indicated in the right of the panel.

or 250 mM NaCl (Fig. 1B, lanes 10, 11, 13, and 14). In addition, in the presence of 500 mM NaCl, hB23.1 hardly binds to histone H2A/H2B complexes but shows the trace level of binding activity of histone H3/H4 complexes (lanes 15–17). These observations suggest that hB23.1 preferentially binds to histone H3/H4 complexes. In an attempt to identify which histone, H3 or H4, or both, is a target of hB23, far-Western blotting was carried out using ³²P-labeled hB23.1 as a probe (see Section 2). Increasing amounts of core histones were separated on a 18% SDS–PAGE, transferred to a PVDF membrane, and then probed with ³²P-labeled hB23.1 (Fig. 2B). Only a band corresponding to histone H3 is detected (Fig. 2B, lanes 4–6), indicating that hB23.1 specifically binds to histone H3.

Next, a histone binding domain(s) of hB23 were determined also by far-Western blotting using ³²P-labeled core histones as a probe. Several mutant hB23 proteins (Fig. 2A) were separated on a 12.5% SDS–PAGE and stained with CBB (Fig. 2C,

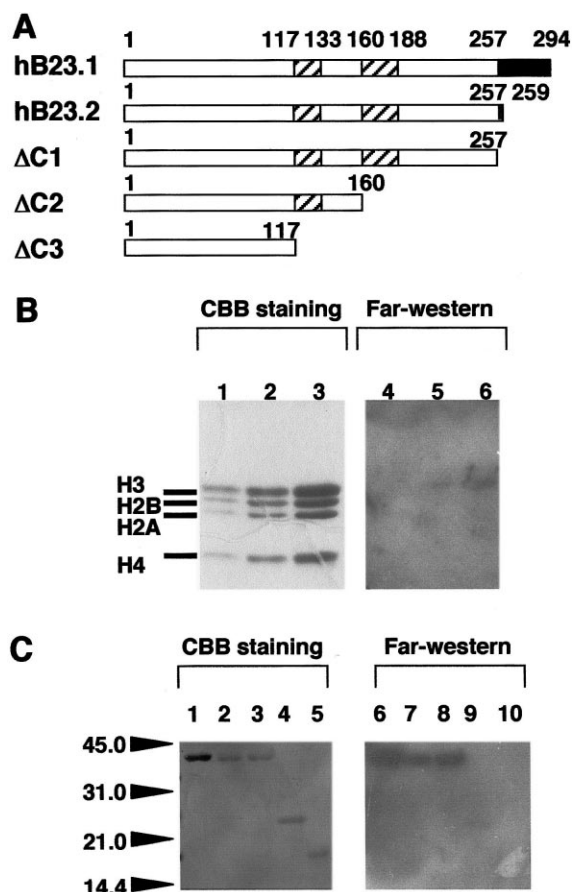


Fig. 2. Histone binding domain of hB23 proteins. A: Schematic diagram of recombinant wild-type and mutant hB23 proteins. The specific regions for two subtypes were shown in black or gray boxes, and the acidic regions were shown by shaded boxes. B: hB23 binds preferentially to histone H3. Increasing amounts of core histones (0.3, 1, and 3 μ g for lanes 1 and 4, 2 and 5, and 3 and 6, respectively) were separated on a 18% SDS-PAGE and stained with CBB (lanes 1–3) or transferred to a PVDF membrane followed by far-Western blotting using 32 P-labeled rhB23.1 as a probe (lanes 4–6). Positions of histone H2A, H2B, H3, and H4 are indicated at the right side of the panel. C: Histone binding domain of hB23 protein. Recombinant wild-type and mutant hB23 proteins (500 ng each) as indicated were separated on a 12.5% SDS-PAGE followed by staining with CBB (lanes 1–5) or far-Western blotting using 32 P-labeled core histones as a probe (lanes 6–10). The positions of molecular weight markers are indicated at the left side of the panel.

left panel) or transferred to a PVDF membrane followed by incubation with 32 P-labeled core histones (Fig. 2C, right panel). Both hB23.1 and hB23.2 are bound by core histones, and ΔC1 lacking the specific region for each subtype is also detected (Fig. 2C, lanes 6–8). This result suggests that the specific region of hB23.1, which is suggested to be involved in ribosome biogenesis, is dispensable for the histone binding activity. In contrast, the histone binding activity of ΔC2 which lacks an acidic region proximal to the C-terminus is significantly decreased, and that of ΔC3, a protein lacking both acidic regions, is virtually unbound by core histones (Fig. 2C, lanes 9 and 10). This suggests that the acidic regions of hB23 proteins are crucial for the histone binding activity. Thus, the results shown in Figs. 1 and 2 altogether support the idea that hB23 is a histone binding protein.

We next investigated using the supercoiling assay whether hB23 mediates nucleosome assembly (Fig. 3A). This assay is

based on the fact that negative supercoil is introduced by assembly of the chromatin structure on relaxed open circular DNA in the presence of topoisomerase I. Supercoiled DNA, which can be separated from relaxed DNA by electrophoresis on an agarose gel, is recovered by removal of chromatin proteins. Core histones preincubated in the absence or presence of hB23 proteins were mixed with relaxed open circular plasmid DNA, and further incubated in the presence of topoisomerase I. Supercoiled DNA is not generated in the absence of chaperone proteins (Fig. 3A, lanes 1 and 8), while rTAF-I β introduces supercoil (lanes 2 and 3) as previously reported [9]. In the presence of either rhB23.1 or rhB23.2, supercoiled DNA is generated in a dose-dependent manner (Fig. 3A, lanes 4–7). This result suggests that hB23 proteins bind to histones and transfer them to plasmid DNA to assemble the nucleosome structure. Since supercoiled DNA was not generated when the same experiment was performed in the absence of core histones, hB23 proteins themselves do not have the activity to introduce supercoil into the plasmid DNA (data not shown). To further confirm whether generation of superhelical

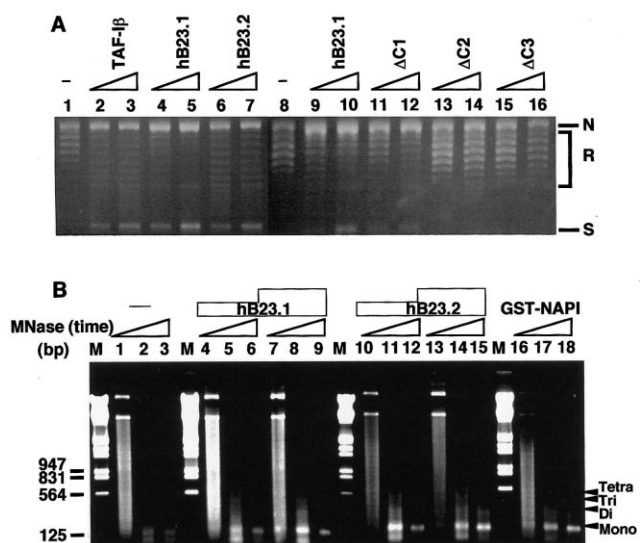


Fig. 3. Nucleosome assembly activity of hB23 proteins. A: Supercoiling assay. The nucleosome assembly activity of rhB23 proteins was examined by supercoiling assay. Core histones (200 ng) preincubated without (lanes 1 and 8) or with 100 ng (lanes 2, 4, 6, 9, 11, 13, and 15) or 300 ng (lanes 3, 5, 7, 10, 12, 14, and 16) of recombinant TAF-I β (lanes 2 and 3), rhB23.1 (lanes 4, 5, 9, and 10), rhB23.2 (lanes 6 and 7), ΔC1 (lanes 11 and 12), ΔC2 (lanes 13 and 14), and ΔC3 (lanes 15 and 16), were mixed with closed circular DNA relaxed by topoisomerase I and further incubated. The DNA was purified and separated by electrophoresis on a 1% agarose gel, and visualized by staining with ethidium bromide. Positions of relaxed (R), supercoiled (S), or nicked (N) circular plasmid DNA are indicated. B: MNase digestion assay. Nucleosome assembly reactions were carried out using core histones (500 ng) preincubated without (lanes 1–3) or with 500 ng (lanes 4–6, and 10–12) or 2 μ g (lanes 7–9, and 13–18) of recombinant hB23.1 (lanes 4–9), rhB23.2 (lanes 10–15), and GST-NAP-I (lanes 16–18), and relaxed plasmid DNA (500 ng). Then, the concentration of CaCl₂ was adjusted to 3 mM and DNA was digested by MNase (1 U) at 37°C. Each histone–DNA complex was digested for 15 (lanes 1, 4, 7, 10, 13, and 16), 90 (lanes 2, 5, 8, 11, 14, and 17), or 480 s (lanes 3, 6, 9, 12, 15, and 18). DNA was purified, separated on a 1.6% agarose gel electrophoresis, and then visualized by staining with ethidium bromide. The positions of molecular weight markers and the bands corresponding to mono-, di-, tri- and tetranucleosome were indicated at the left and right sides of the panel, respectively.

DNA is due to nucleosome formation, micrococcal nuclease (MNase) digestion assays were carried out (Fig. 3B). After the nucleosome assembly reaction, histone–DNA complexes were subjected to digestion by MNase. As a control experiment, NAP-I tagged with GST, a well-characterized histone chaperone, was also tested for the ability of nucleosome formation (Fig. 3B, lanes 16–18). MNase-resistant DNA of approximately 150, 300, or 450 bp corresponding to mono-, di-, or trinucleosome are detected when the nucleosome assembly reaction is performed in the presence of NAP-I and treated with a low amount of MNase (lane 16). By treatment with higher amounts of MNase, a distinct DNA band corresponding to mononucleosome is generated in NAP-I-dependent reactions (lanes 17 and 18), while only a trace amount of mononucleosomal DNA is seen in the absence of NAP-I (lanes 2 and 3). The digestion pattern of the assays in the presence of either hB23.1 (lanes 4–9) or hB23.2 (lanes 10–15) is quite similar to that of NAP-I, suggesting that the activity of hB23 proteins is comparable to that of GST-NAP-I under the experimental condition employed here (compare lanes 7–9 and 13–15, and 16–18). Recombinant mutant hB23 proteins (see Fig. 2A) were also tested for the nucleosome assembly activity. The activity of $\Delta C1$ is comparable to that of wild-type hB23.1 (Fig. 3A, lanes 9–12). However, $\Delta C2$ and $\Delta C3$ proteins lacking the acidic regions are not efficiently capable of mediating nucleosome formation (Fig. 3A, lanes 13–16). This result is closely correlated with the histone binding activity. It is noteworthy that nucleosome structure assembled only by a histone chaperone protein has been shown incomplete. A spacing factor such as ACF is required to generate periodical nucleosome arrays [4]. This was indeed the case for the nucleosome structure assembled by hB23 proteins and NAP-I (Fig. 3B).

Finally, we tested hB23 for the sperm chromatin decondensation activity (Fig. 4). As previously reported, histone chaperone proteins such as nucleoplamin, NAP-I, DF31, and TAF-I decondense densely packed demembrated sperm chromatin [8,17,18]. These histone chaperone proteins are associated with sperm-specific basic proteins or histones, resulting in remodeling the sperm chromatin. The activity to mediate decondensation of demembrated *Xenopus* sperm chromatin is a sensitive tool to test a protein for the histone chaperone activity. Demembrated *Xenopus* sperm chromatin was incubated without or with recombinant proteins, and chromatin DNA was visualized by staining with Hoechst 33258. Either *Xenopus* egg extracts or recombinant TAF-I was shown highly active [8]. Both subtypes of hB23 proteins also decondense sperm chromatin (Fig. 4). The sperm chromatin decondensation activities of recombinant hB23 proteins are slightly lower than those of egg extracts and TAF-I (data not shown). $\Delta C1$ lacking the C-terminal unique sequence for hB23.1 and hB23.2 also decondenses sperm chromatin comparable to wild-type hB23 proteins. The activity of $\Delta C2$ is much lower than those of wild-type proteins, and $\Delta C3$ fails to decondense sperm chromatin. It turns out that the acidic regions of hB23 proteins are required for the adenovirus chromatin remodeling, histone binding, and nucleosome assembly assays.

We show here that hB23 proteins function as histone chaperones in several in vitro assays. Since hB23.1 is co-precipitated with chromosomal histones and topoisomerase II from cell extracts [10], it is possible that hB23.1 is bound to chromatin in cells. Interestingly, it was reported that B23.1 binds

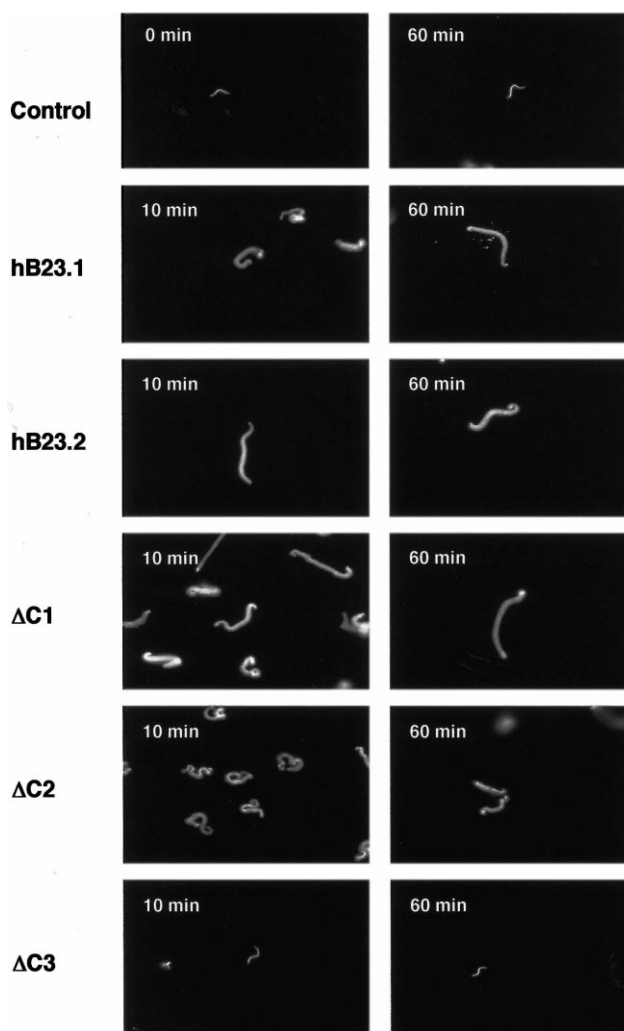


Fig. 4. hB23 decondenses sperm chromatin. Demembrated *Xenopus* sperm chromatin (5×10^4 sperm equivalents) was incubated with 5 μ g of recombinant proteins as indicated for 10 min (left column) or 60 min (right column) followed by fixation with formaldehyde. DNA was stained with Hoechst 33258 and visualized under a fluorescent microscope. Sperm chromatin incubated without hB23 proteins for 0 min or 60 min is shown at the top panels as controls.

to retinoblastoma protein and enhances the DNA polymerase activity in vitro [19]. These observations lead to a speculation that hB23 plays some roles during DNA replication. Unlike CAF-I and ASF-I in RCAF complex, the association between hB23 proteins and free histones in cell extracts has not been observed under our experimental conditions. During transcription and replication, nucleosome structure could be remodeled partly by removing histones from chromatin. This idea is supported by the result that a multisubunit–nucleosome remodeling complex, RSC, was shown to remove nucleosomal histones and transfers them to naked DNA in *trans* [20]. In this case, it is possible that hB23 functions transiently as an acceptor of histones which are removed during transcription or replication to remodel the chromatin structure. After these reactions, hB23 could re-deposit histones to DNA to assemble the chromatin structure.

Although functional importance of the N-terminal portion of hB23 proteins has not been evaluated here, at least the acidic regions of hB23 proteins are critical for their histone

chaperone activity. The N-terminal portion of hB23 proteins shows significant similarity to nucleoplasmin family proteins and was shown to be important for oligomer formation [21]. From these observations, it is presumed that the N-terminal region of hB23 is important for proper folding of the protein. On this line, we previously showed that the acidic region of TAF-I itself, acidic molecules such as RNA, and mutant TAF-I proteins that fail to form dimer but contain the acidic region are not functional as histone chaperones [8,10,22].

The results shown here emphasize that B23 contains acidic regions that are functionally important for its putative role in regulating chromatin structure. Thus, B23 proteins are proposed to be categorized into a family of 'acidic molecular chaperone' [3] members for the basic viral core proteins and cellular histones.

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