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We have studied the sample concentration-dependent and external stress-dependent stability of native and reconstituted nucleosomal arrays. While upon stretching a single chromatin fiber in a solution of very low chromatin concentration the statistical distribution of DNA length released upon nucleosome unfolding shows only one population centered around ~25 nm, in nucleosome stabilizing conditions a second population with average length of ~50 nm was observed. Using radioactively labeled histone H3 and H2B we demonstrate that upon lowering the chromatin concentration to very low values first the linker histones are released, followed by the H2A-H2B dimer, while the H3-H4 tetramer remains stably attached to DNA even at the lowest concentration studied. The nucleosomal arrays reconstituted on 5S rDNA tandem repeat exhibited similar behavior. This suggests that the 25 nm disruption length is a consequence of the histone H2A-H2B dimer dissociation from the histone octamer. In nucleosome stabilizing conditions full ~145 bp is constrained in the nucleosome. Our data demonstrate that the nucleosome stability and histone octamer integrity can be severely degraded in experiments where the sample concentration is low.

The genomic DNA of all eukaryotes is packed in the cell nucleus in the form of chromatin. It has been well established that beside compaction of the important amount of genomic DNA in the restricted nuclear volume, the chromatin organization is the key element in the control of gene expression (e.g. (1,2). Its most abundant part is represented by association of the DNA with a specific class of proteins - the histones, forming a basic repetitive unit of the chromatin - the nucleosome. 145 bp of DNA is associated in ~1,65 left-handed turns with the histone octamer, composed of two copies of each of histones H2A, H2B, H3 and H4, to form the Nucleosome Core Particle - NCP (3). Linker DNA interconnects NCPs and is usually associated with the linker histones. The complex of the histone octamer, linker histone and 166 bp of nucleosomal DNA is termed chromatosome (3). Consecutive nucleosomes tend to organize into ~30 nm thick irregular fiber, whose level of compaction and organization alters with ionic conditions and the presence of linker histones and core histone N-terminal domains (4-8).

The gene regulation function of the chromatin is directly related to mechanisms underlying the chromatin conformation changes that involve, among others, the nucleosome stability and its structural alterations linked to nucleosome remodeling mechanisms. Because of this, the elasticity of the chromatin fiber and of individual nucleosome has become an intensively studied topic over the last few years.

Measuring the elastic properties of chromatin arrays using optical tweezers is one of the most direct methods assessing the forces involved in the nucleosome stability and chromatin fiber organization. Several studies were recently reporting on the elastic response of the chromatin fiber to stretching forces. Traction of chromatin segments assembled in nuclear extract of *Xenopus laevis* yielded a 'saw tooth' pattern stretching profile with typical disruption length of 65 nm at forces around 20 pN (9). Similar force value was observed for nucleosomal arrays reconstituted on a tandem repeat of 5S rDNA sequence (without linker histone) and the length of DNA released upon the nucleosome disruption was found to be 27 nm (10). Surprisingly, neither of these values corresponds to those expected from the known NCP and chromatosome structure. The first contains ~49 nm of DNA (145 bp) in contact with histone octamer and 56 nm (~166 bp) of DNA are in contact with the histones within the chromatosome. It was proposed (11) that the observed 65 nm disruptions in nuclear extract experiments may reflect the binding of both B4 (the embryonic form of linker histone) and high mobility group (HMG) proteins to chromatin.

The experiments with 5S reconstituted arrays suggested (10) that the DNA unwrapping from the H2A-H2B dimer occurred progressively in low force regime (~5 pN) and the major disruption event was associated with the dissociation of the remaining DNA from the H3-H4 tetramer. This is in good agreement with the ~80 bp of unwrapped DNA i.e. the 27 nm observed (10). Taking in account the distribution and type of DNA-histone bonds within the NCP (12) it is, however, difficult to justify the ~threefold difference in threshold forces for H2A-H2B dimer and H3-H4 tetramer respectively. Moreover, the force values necessary for the total nucleosome disintegration by far exceeds the theoretical estimates (13) and results obtained by thermodynamic considerations (14-16). In order to deal with these controversies, several explanations were proposed. Kulic and Schiessel (17) suggested, that the major part of nucleosome disruption energy is absorbed by flipping of the NCP around its dyad and only a minor part is consumed by the DNA unwrapping. This can potentially equilibrate the contribution from the H2A-H2B dimer and the H3-H4 octamer and bring the unwrapping energy down to the predicted values. Another proposition (18) pointed out the possible effect of the loading rate. Removal of the whole octamer likely needs to pass over a large energy barrier, which can be achieved by thermal fluctuations only on long time scales. It is therefore possible, that the stretching velocities so far exploited are much too fast, bringing the system far from its thermodynamic equilibrium.

This work reports our data on the nucleosome unfolding upon external stress and sample dilution. We have studied and compared the elastic behavior of native chromatin isolated from chicken erythrocytes and nucleosomal arrays reconstituted on tandem repeat of 5S rDNA sequence. In order to establish the contribution of the H3-H4 tetramer we also investigated the elastic response of the chromatin segments reconstituted with histone H3-H4 tetramer only. Our data allows to understand in depth the mechanism of nucleosome unfolding upon applying external forces and to clarify the contradictions in the literature.

Materials and methods

Reconstitution of 5S nucleosomal arrays

Plasmid p2085S-G5E4, (kind gift from J. L. Workman) was restricted with *Acc*65I (*Asp*718): G*GTAC*C, *Cai*I (*Alw*NI), *Ssp*I, ClaI: AT*CG*AT (Fermentas) and restriction fragments were purified by 4% native PAGE and electro-elution and quantified by absorbance at 260 nm. The 2539 bp long fragment consists of a ~ 400 bp E4 promoter DNA, flanked by two DNA sequences each containing five 208 bp tandem repeats of the 5S rRNA sea urchin gene (19). Purified fragments were end-labeled either at the *Cla*I side by biotin-dCTP, or [α-^{32}P] dCTP in presence of 100 µM of dGTP using exo$^-$ Klenow fragment. A fraction of biotin-dCTP labeled fragments was labeled at the Acc65I end by digoxigenin-dUTP (Roche). Conventional recombinant *Xenopus laevis* histones were expressed in bacteria and purified according to the protocol of Luger et al. (1999) (20). For reconstitution the end-labeled DNA fragments were mixed in 2 M NaCl with a 1:0.8 molar ratio of the recombinant core histones and reconstituted by salt dialysis as described in (21). Typically 1 µg of biotin labeled DNA fragments, containing 10-16% of doubly (biotin - digoxigenin) labeled fragments were used for reconstitution. For the

Micrococcal nuclease (MNuc) digestion analysis of the nucleosomal arrays, ^{32}P- labeled fragments were used instead. 100 ng of ^{32}P end-labeled free DNA and nucleosomal array were partially digested with 0.03 and 0.1 U of MNuc, respectively in presence of 1 mM Ca^{++} for 2'. The reaction was stopped by 100 µl of 0.2% SDS and 1 µg of Proteinase K. DNA was extracted by phenol-chlorophorm, ethanol precipitated and run on 1.5% agarose gel. The *Eco*RI ladder was obtained by partial digestion of DNA with 2 units of *Eco*R1 for 1' (line 1). Positions of nucleosomes within 5S-5GE4 array are indicated.

Native chromatin preparation

The isolated chicken erythrocyte nuclei were washed twice with digestion buffer (60 mM KCl, 15 mM NaCl, 15 mM PIPES pH 7.5, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 0.1 mM AEBSF). Micrococcal nuclease (Fermentas) was added to final concentration 1 U/1 OD_{260} of DNA and nuclei digested for 1' at 37°C. The reaction was stopped by adding EDTA to 5 mM final concentration. Nuclei were centrifuged at 400 g at 4°C for 10' and the supernatant discarded. Pellet of nuclei was re-suspended in 1mM EDTA and left on ice for 45' to release the chromatin. The sample was centrifuged 10' at 5000 g and the supernatant containing chromatin removed and placed into new tubes. This procedure yielded chromatin segments ranging from 2 kb to ca 15 kb with the highest proportion around 7 kb and low amount of short oligonucleosomes. For biotinylation, the chromatin was washed twice in 25 mM Na phosphate, 0.5 mM $MgCl_2$, pH 7.5 using Microcon YM 100 tubes (400 g, 25', 4°C). One unit of T4 DNA polymerase per 1 µg of DNA was added to solution and incubated 10' at 37°C to form 3' overhangs. The mix of nucleotides (biotin14-dCTP, biotin14-dATP, dGTP and dTTP) was added to the reaction to final concentration of 100 µM each and incubated 15' at room temperature. The reaction was stopped by adding EDTA to 5 mM final concentration. The sample was placed into 1x TE (10 mM Tris, 1mM EDTA) buffer using Microcon YM 100 concentrator.

Nucleosome dissociation experiments

For the nucleosome dissociation experiments a wild type H3 and swapped tail H3-H2B mutant histones were used. The swapped tail H3-H2B is a fusion between the N-terminal tail of histone H3 and the histone fold domain of H2B (22). Both the wild type H3 and the fusion H3-H2B were radioactively labeled using Aurora A kinase (23). End-positioned nucleosomes contained labeled either H3 or the fusion H3-H2B and the H3-H4 tetrameric particles were reconstituted on a non-labeled 241 bp fragment comprising the 601 positioning sequence as previously described (22). Nucleosome dissociation experiments were carried out in either TE, 10 mM NaCl or in PBS. Briefly, aliquots of nucleosomes were diluted with the appropriate buffer in 10 µl final volume to the concentrations indicated (40-5 nM), and left for 45 minutes at room temperature. Then the samples were analyzed by Electrophoretic Mobility Shift Assay (EMSA) carried out in 5% (w/v) polyacrylamide gel (acrylamide to bisacrylamide, 29:1 w/w), 0.3x TBE at 4°C (21).

For the native chromatin dissociation experiments, 2 to 4 µg of chromatin at a concentration 0.25 mg/ml (measured as dsDNA OD_{260}) were diluted in appropriate volumes of 1x PBS to the concentrations as indicated, and left at room temperature for one hour. Then, $MgCl_2$ was added (final concentration 15 mM) to the chromatin samples and they were additionally incubated for 5 minutes on ice to complete the $MgCl_2$-induced chromatin aggregation. The aggregated chromatin samples were quantitatively pelleted by centrifugation at 16000 g for 15 minutes at 4°C. Each pellet was dissolved in 15 µl of 1x loading buffer for SDS electrophoresis and the samples were loaded on 18% polyacrylamide gel containing SDS. After completing the electrophoresis the gels were stained with coomassie.

Stretching experiments

In stretching experiments an experimental flow cell with micropipette was used similar to that described in (24). Reconstituted chromatin fragments (OD_{260} = 0.5) were first incubated overnight at 4°C with 0.005% w/v of solid matter streptavidin (Amersham) functionalized beads (Spherotec, 3.36 µm) in 1x PBS, pH 7.5 in total volume of 50 µl. The beads were functionalized according the standard protocol for COOH activated beads (25). A solution of digoxigenin (Roche) functionalized 1.5 µm Sicastar® bead (Micromod) were introduced into the experimental

cell and one fixed by suction in the micropipette. The solution of beads incubated with chromatin was 100x diluted in 1x TE, 100mM NaCl and introduced into the experimental cell (the additional dilution was necessary in order to lower the bead concentration to a level compatible with the stretching experiment). A selected bead was immobilized in the optical trap and by approaching the bead held in micropipette the chromatin fragment was tethered between the two beads. For native chromatin, the tethering procedure was identical with the exception of using both beads (large and small) activated with streptavidin. The chromatin fragment and bead concentration were adjusted depending on the experimental conditions. Typical data acquisition frequency was 3 kHz and weak averaging was applied in order to increase signal/noise ratio. The speed of the traction was 100 nm/s with trapping constant 70 pN/µm. Lower speeds down to 10 nm/s were applied to study the effect of loading rate. For each material and experimental conditions a large statistical set of data was collected and analyzed (number of disruption ~500 for native chromatin, ~460 for reconstituted chromatin, corresponding to ~50 stretched segments for each, ~80 for reconstituted tetramers and ~120 for native chromatin in presence of $MgCl_2$) in order to obtain a representative value. For optimized conditions, the above protocol was modified in order to preserve the native chromatin concentration sufficiently high throughout the whole procedure to prevent the concentration driven histone octamer dissociation. A relatively high quantity of beads (0.05% solid w/v final for 3.36 µm beads) was added to the chromatin at OD_{260}>4 and left incubate overnight at 4°C. Prior the stretching, the solution was diluted in order to obtain the same conditions (bead concentration and ionic conditions) as above and stretching experiment completed.

The length of DNA released upon the individual nucleosome unfolding was evaluated similarly as described in (9). Statistical sets were fitted with a Gaussian (or linear combination of Gaussian) using Kaleidagraph data analysis software.

RESULTS

Elasticity of reconstituted 5S rDNA nucleosomal arrays

Nucleosomal arrays were reconstituted by using core histones purified to homogeneity (Fig. 1A) and a fragment of DNA containing 208 bp repeats comprising the 5S rRNA sea urchin gene nucleosome positioning sequence. This allows to reconstitute, under optimal conditions, arrays containing twelve nucleosomes (19). The reconstituted arrays were analyzed by digestion with micrococcal nuclease (Fig. 1B). The clear ~200 bp repeat observed upon micrococcal nuclease digestion evidences for a proper organization of the reconstituted samples. Once the arrays were characterized, we carried out the elasticity measurements. The force/extension curve showed typical 'saw tooth' profile (Fig. 2A) similar to the one described earlier, where the discontinuities in elastic response correspond to the unfolding of individual nucleosomes (10). Contrary to previously published data, we have, however, observed rather large spread of threshold forces, with the average at the position of 17.6 ± 3.5 pN (Fig. 2B inset and Table I). Although there were cases, where nearly all disruptions occurred at approximately the same force, we believe that certain spread of values is normal and we interpret it as a natural fluctuation of the nucleosome conformation. The statistical distribution of the DNA length released upon the nucleosome disruption showed a main, very narrow peak around a central value of 24.1 ± 3.9 nm (Fig. 2B). In addition, some longer disruptions were observed, which is expressed by a very weak shoulder in the statistical distribution (Fig. 2B) with the central value of 47.5 ±5.4 nm. The main peak at ~24 nm was described previously in (10) and was attributed to the unwrapping of the central 80 bp nucleosomal DNA stabilized by the interactions with the H3-H4 histone tetramer (10). The shoulder at ~50 nm was not reported in the literature and its origin was unknown.

We have also evaluated the potential effect of divalent ions as it might potentially have an effect on the histone octamer conformation. The addition of $MgCl_2$ (to 1 mM final concentration) has no effect on the stretching profile of reconstituted segments and the statistical distribution of disruption lengths remains identical (Fig. 2B).

Elasticity measurements of native chromatin

The reconstituted chromatin arrays, although well defined, represent only an incomplete substitute of the native chromatin. The presence/absence of the linker histone may significantly modify the stability of the entire nucleosome or alter eventually the stability of the histone octamer. We have therefore decided to study the elastic response of native chromatin. High molecular weight native chromatin segments were isolated from chicken erythrocytes nuclei by mild digestion with micrococcal nuclease (see Materials and Methods). The native chromatin contained a full complement of the core as well as linker histones (data not shown but see Fig. 6). The stretching experiments were carried out under identical experimental conditions as in case of reconstituted nucleosomal arrays. For the native chromatin the disruption discontinuities varied both in threshold force and length of released DNA (Fig. 3A). The distribution of disruption lengths is clearly biphasic comprising a main peak and a well pronounced shoulder (Fig. 3B). Although the disruption length varies rather widely, the statistical evaluation reveals the main peak exhibiting a value of 23.2 ±3.5 nm (Fig. 3B and Table I), while the average length of the unwrapped DNA of the shoulder corresponds to 40.1±5.5 nm (Fig. 3B). Therefore, the dominant value of the DNA length released upon the disruption is identical (within the error margin) to the one obtained for the reconstituted chromatin (Table I, Fig. 2B). In addition, the average measured value of the disruption force was 19.6 ± 4.6 pN (Fig. 3B inset and Table I) which is equal, within the error, to that measured for the reconstituted chromatin segments.

Stretching experiments with nucleosomal arrays reconstituted with H3-H4 tetramer only

It was suggested earlier (10) that the ~24 nm disruption results from unfolding the central 80 bp of DNA interacting with the H3-H4 tetramer. If this was correct, the distribution of disruption lengths of an array of reconstituted H3-H4 tetramers should be identical to that of the complete octamer. To test if this was the case, we have reconstituted 5S arrays of H3-H4 particles and have studied their elastic properties. The stretching profile of these samples was very similar to that of the arrays reconstituted with the complete core histone octamer (results not shown). The average disruption length was found again ~25 (25.1 ± 4.8) nm. However, contrary to the native segments or reconstituted chromatin, the disruption length distribution contained no long disruptions (Fig. 4) and they were completely absent in the stretching profiles. The average measured force was 14.7 ± 1.8 pN, i.e. slightly lower than in case of arrays reconstituted with the whole histone octamer, but still within the error margin of its average threshold force (17.8 ± 5.5 pN, see Table I). All these data suggest that the peak at ~24 nm might really reflect the unwrapping of the central 80 bp of nucleosomal DNA bound by the histone H3-H4 tetramer. Bearing in mind this and the fact that the 24 nm peak was the major peak in the disruption length distribution of both native chromatin and arrays reconstituted with histone octamers, one could think that there might be no contribution of H2A-H2B dimer or of the linker histone to the stability of the nucleosome.

Native chromatin and reconstituted nucleosomal array stability at very low sample concentrations

Why there is no detectable contribution of H2A-H2B dimer or linker histone to the stability of the nucleosome? One explanation is that the binding forces and energies of the H2A-H2B dimer are much weaker than those of the remaining tetramer and therefore their contribution to the stretching profile is not expressed in the form of apparent discontinuities, but rather in form of nearly continuous DNA unfolding at low force values (10). However, as mentioned above, the overall distribution of the DNA histone contacts as revealed by the crystal structure cannot easily explain the nearly threefold increase in the threshold force for the H3-H4-nucleosomal DNA complex and especially the all-or-none mode of the nucleosome disintegration.

An alternative explanation could be suggested. Detailed studies on the stability of the NCPs within a large interval of the monovalent ion concentrations (10^{-4}-2 M) were reported (15,26). The data showed that the mechanisms of histones release from the NCPs involved: (i) a highly cooperative release of the histones from the DNA, consisting of complete eviction of the histone octamer from DNA; this mechanism is realized at NaCl concentrations < 0.75 M and, (ii) a stepwise release of histones from the NCP's DNA at NaCl

concentrations > 0.75 M, the histone H2A-H2B dimers being released more readily than the histone H3-H4 tetramers (27). This leads to a heterogeneous population of intact NCPs and particles, which contain the H3-H4 tetramer only.

It has been also observed that at very dilute solution NCPs exhibit considerable destabilization already at physiological salt concentration (16,28). Therefore, the stability of the NCP in solution depends on the concentration of both the monovalent (M^+) ions and the NCPs themselves (29). These two mechanisms were shown to operate at NCP concentrations above 5μg/ml (29). To our knowledge no data are available below this limit concentration of NCPs due to difficulties in the detection of the small amount of released histones. In the case of chromatin stretching experiments the concentration of the samples was far below than 5μg/ml (see Materials and Methods). We hypothesized that at very low NCP concentration a selective release of the H2A-H2B dimer might occur and the studied samples could contain mainly H3-H4 tetramers. This could explain why upon stretching of chromatin mainly 80 bp DNA is unfolded.

The main difficulty when studying the potential release of histones from NCPs at low particle concentration is, as noted above, the detection of a very small amount of released protein. We have overcome this by using NCPs reconstituted with ^{32}P-labeled histones (see Materials and Methods). Briefly, histones H2B and H3 were ^{32}P-labeled and were used to reconstitute end positioned nucleosomes on a 241 bp DNA fragment containing the 601 positioned sequence (30). The nucleosomes contained labeled either histone H2B or histone H3. H3-labeled tetrameric H3-H4 particles were also reconstituted. Then the Electromobility Shift Assay was used to study the behavior of these particles upon successive twofold dilutions in the range of 40-5 nM NCP concentrations at low ionic strength (1x TE, 10 mM NaCl) and in PBS (~ 160 mM Na^+) (Fig. 5). It should be noted that identical volume of individual samples was loaded on the gel and thus each successively loaded sample contained twofold lower radioactivity than the previous one (Fig. 5). If upon dilution the H2A-H2B dimer is released from the H3 labeled NCP a second band with lower electrophoretic mobility corresponding to the H3-H4 tetramer should be observed in addition to the NCP band. No such band would be detected for particles with labeled H2B, since there is no label in H3-H4 tetramer. Only a decrease of the octamer band intensity should be seen instead due to both the dilution and the possible release of the labeled H2A-H2B dimer. This is, indeed, the case (Fig. 5). The effect, as expected, is much better pronounced for the particles in higher ionic strength where in 1x PBS already at 40 nM NCP concentration a band corresponding to the H3-H4 tetramer is clearly seen (Fig 5, 1x PBS, lane 1') and at the lowest concentration (5 nM) the NPC completely disappeared and only the H3-H4 tetrameric band is observed (Fig. 5, 1x PBS, lane 4') In low ionic strength (TE, 10 mM NaCl) the NCP remained relatively stable down to ~10 nM concentration (compare lanes 1-4 with lanes 1'-4', respectively). We conclude that at very low NCP concentration, the H2A-H2B dimer dissociates from the particles (particularly at higher ionic strength) and thus the samples contained mainly H3-H4 tetramers. Accordingly, since the stretching experiments were carried out at much lower concentration of nucleosomal arrays (see Materials and Methods), the disruption should reflect mainly the unfolding of the tetrameric H3-H4 particle. We hypothesized that the peak at 25 nm in the disruption length distribution results from the unfolding of the tetrameric H3-H4 particle, while the small shoulder at around 50 nm would reflect disruptions of very rare intact NCPs (see Fig. 2B).

It was noted that addition of non-ionic detergents at low concentration improves assembly of NCPs and often they are added to the working buffers (e.g. (10). The addition of NP40 to 0.03% v/v concentration indeed significantly improved the NCP stability in intermediate dilutions, but its effect disappeared at lowest ones, particularly in physiological salt concentrations (data not shown).

The disruption length distribution of native chromatin showed a much better pronounced shoulder of longer values (Fig. 3B, Table I) suggesting that native chromatin is more stable upon dilution compared to reconstituted nucleosomal arrays. This stabilization could be due to either the presence of linker histones or to the better assembly of native chromatin or to both. To study the behavior of native chromatin in 1x PBS at very low concentrations, chromatin dilution experiments similar to these for NCP were performed (Fig. 6). Following dilution with a

solution of 1x PBS the different chromatin samples were precipitated and their histone composition analyzed by SDS gel electrophoresis (see Materials and Methods for details). As seen, upon dilution of chromatin the intensity of the bands corresponding to the linker histones H1 and H5 drastically decreased (fig. 6A, compare lanes 1 and 2 with lanes 4-7, and figure 6B) and already at 1.25 nM they were difficult to be detected on the gel (Fig. 6, lane 8). On the contrary, the amount of H2A and H2B remained constant down to 5 nM (where the bands of linker histones were very weak) and then began to decrease (Fig. 6 B, compare the scans for 5 nM with this of 2.5 nm). Therefore, upon dilution a release of H1 is first realized and then the H2A-H2B dimer is released from chromatin DNA. This agrees well with rather high histone H1 mobility observed *in vivo* (31) suggesting loose binding of linker histone to the chromatin. Since the stretching experiments were carried out at concentrations lower than 1 nM (see Materials and Methods) they should be associated with the disruption of heterogeneous particle population containing either whole octamer or H3-H4 tetramer only. Consequently, the distribution of disruption length would reflect the unwrapping of the H3-H4 tetramer particle (the peak at 24 nm) and the intact NPC (the shoulder at around 50 nm), respectively. Since the stability of the native octamer particle is higher compared to the reconstituted octamer particle (the concentration at which the H2A-H2B dimer begins to dissociate from the native chromatin is around 1 nM compared to ~40 nM for reconstituted nucleosomal arrays, Figs. 5 and 6), one should expect a higher proportion of 50 nm disruptions in the native chromatin than in reconstituted samples, which is indeed the case.

Elastic response under optimized conditions

Since the histone dissociation increases dramatically with chromatin dilution, increasing chromatin concentration should move the equilibrium towards more stable histone octamer. Thus, the presence of exogenous chromatin sample would stabilize the chromatin fibers prepared to be stretched and one should expect the formation of higher amount of intact particles and an increase of the shoulder at 50 nm. To test this, we have added to the solution of the labeled 5S reconstituted arrays a non-labeled exogenous chromatin at a concentration of 100 μg/ml. Although the effect was not dramatic, the distribution of the disruption length in the presence of the exogenous samples exhibited a clear increase of the longer distributions centered around 50 nm corresponding to the intact NCP containing 145 bp of DNA (Fig. 7).

The effect of increasing the overall chromatin concentration on the stretching profile of native chromatin was much better pronounced (Fig. 8A) especially when performing the chromatin tethering under optimized conditions (see Materials and Methods). The most striking feature was the high proportion of long disruption lengths, which was far higher than in any of the previous experiments. The statistical distribution (Fig. 8B) clearly showed two major peaks located at 24 and 50 nm, having almost the same statistical weight. We would like to note that an increase of the 50 nm peak was also observed upon stretching the chromatin at lower ionic strength (which tends to stabilize the nucleosome), even without adding exogenous samples (results not shown, but see Table I where the parameters of the stretching profile for different experimental conditions are presented).

DISCUSSION

This work reports our data on the stability of reconstituted nucleosomal arrays and native chromatin. We have shown that at very low sample concentration a stepwise dissociation of histones takes place. For native chromatin, upon decreasing the sample concentration, the first histone to be released is the linker histone (H5 and H1), followed by the H2A-H2B dimer. The H3-H4 tetrameric particle remains quite stable even at very low sample concentrations. The picture for the reconstituted nucleosomal arrays was very similar with the dimer H2A-H2B being released upon the lowering of the sample concentration. These findings are coherent with the observed dilution-driven NCP dissociation (32). It was demonstrated that after very high dilution the consequent re-concentration would not result in NCP re-formation (or, depending on the buffer, its efficiency would be at least strongly impaired) (32). Since the single chromatin fiber stretching experiments are performed typically at very low sample concentrations (0.1-0.5 nM NCP), the

above findings allowed us to understand the mechanism of nucleosome unfolding upon applying external forces. The distribution of the disruption length at very low sample concentration exhibits a main peak centered around 24 nm that we attributed to the unwrapping of ~80 bp DNA, which interact with the H3-H4 tetramer. The second peak, detected at around 50 nm, which is well pronounced when the chromatin segments were stretched under histone octamer stabilizing conditions, was attributed to the disruption of the complete NCP.

The average disruption force was around 20 pN for all chromatin types and conditions studied with the exception of reconstituted tetramers (Table I). This is perhaps a consequence of modified DNA-histone contacts. Very likely the reconstitution with complete octamer followed by the dissociation of H2A-H2B dimer would result in more nucleosome-like structure than in the case of direct reconstitution with H3-H4 tetramer only. The slight difference of ~3 pN between native and reconstituted chromatin is within our error margin and larger data set would be necessary to decide, whether this difference is statistically significant. The disruption length of H3-H4 tetramer reconstituted on 5S is the same (within the error) as for the complete octamer in standard conditions and corresponds to ~80 bp. This value is slightly lower than that determined by other approaches (e.g. (33)) which shows approximately 100 to 120 DNA bp in contact with tetramer. On the other hand, the NCP crystal structure (12) shows at most 70 bp associated with H3-H4 tetramer in intact particle (not considering the isolated DNA contact with H3 proximal part of N-terminus tail at NCP entry/exit site). Also, the space available on the circumference of the H3-H4 tetramer disc is not sufficient to associate with more than ~70 bp. One can speculate, that additional DNA/H3-H4 contacts can be formed non-specifically on the top or bottom surface of the tetramer reaching thus reported alternative values (100-120 bp), but these contacts would be rather weak and would not contribute to the disruption event.

Our study shows, that the elastic response of the H3-H4 tetramer reconstituted on 5S is undistinguishable from the one of complete NCP (with the exception of infrequent longer disruptions in the latter case). Still the question remains, why the nucleosome disruption event occurs in an all-or-none fashion. One would expect to see contributions of individual domains, which is not the case. The most likely explanation would be the argument of Marko (18) that indeed the loading rates are much too fast putting the nucleosome far from its thermodynamic equilibrium. This would also account for the excessive forces needed for the nucleosomal disruption. We have, therefore, checked the influence of the loading rate, by decreasing tenfold the stretching speed in the case of the native chromatin. Even at the stretching speed of 10 nm/s we did not observe statistically significant change either in the length of disruption or the threshold force (data not shown). This would mean that either the influence of loading rate cannot explain for the high energy needed for the NCP unfolding or that even at these rates the system is still far from the equilibrium state. Here it should be noted, that we have noticed slight decrease in threshold force average for very slow stretching from 19 to 15 pN which is unfortunately still within the error bar.

Our data clearly demonstrate that when linker histones were dissociated from chromatin, essentially no H2A-H2B dimers were released. Under optimal stretching conditions, the statistical weight of the peak at 50 nm (attributed to the disruption of an intact NCP) was equal to this of 24 nm that reflects the unwrapping of 80 bp DNA interacting with the histone H3-H4 tetramer. Therefore, about half of the particles to which stretching is applied contain a complete histone octamer. All these considerations suggest that the studied chromatin fibers used for stretching (even under the optimal stabilizing conditions) would not contain detectable amount of the linker histones and the effect of linker histones on stretching profile could not be determined in our studies. Finding conditions that stabilize the association of linker histones with chromatin at low sample concentrations remains a challenge for further studies.

In conclusion, the presented data demonstrate that the single molecule experiments on chromatin fragments present a rather delicate affaire. The instability of the octamer under these conditions is largely enhanced, and can potentially provide misleading results. Clear increase of the disruption length corresponding to entire NCP in conditions

favoring the histone octamer integrity shows the necessity to take this in account.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. **A** - 18% SDS-PAGE analysis of recombinant histones used for reconstitution; **B** - Microccocal nuclease digestion pattern of naked 5S rDNA arrays (lane 2) and reconstituted nucleosomal 5S rDNA arrays (lane 3). The positions of the nucleosomes are designated on the right part of the figure. Lane 1, molecular mass marker.

Fig. 2. Stretching profile of the reconstituted 5S nucleosomal arrays. **A** - Force/extension curve of the stretching cycle – extension in red, relaxation in black; **B** - The distribution of disruption lengths in 1x TE, 100 mM NaCl buffer in absence (shaded bars) and in presence (grey bars) of 1mM $MgCl_2$. Inset, the distribution of the threshold forces (without $MgCl_2$).

Fig. 3. Stretching profile of the chromatin isolated from the chicken erythrocyte nucleus in 1x TE, 100 mM NaCl buffer. **A** - An example of the force/extension curve; **B** - The distribution of disruption lengths. Inset – the distribution of the threshold forces. Note the higher proportion of disruption lengths around ~40 nm compared to this of reconstituted nucleosomal arrays.

Fig. 4. Distribution of disruption lengths of histone H3-H4 tetramers reconstituted on the 5S DNA arrays. The stretching was carried out at 1x TE, 100 mM NaCl buffer. Contrary to the nucleosome arrays reconstituted with histone octamer or to native chromatin (figure 2 and 3), the distribution of lengths entirely misses the longer disruptions.

Fig. 5. EMSA of reconstituted nucleosomes at very low concentrations under different conditions. The nuclesomes contained either ^{32}P-labeled H3 (H3* nucleosomes) or ^{32}P-labeled H2B (*H2B nucleosomes). The tetrameric particles contained ^{32}P-labeled H3 (H3*-H4 particles). On the left part of the figure positions of the intact nucleosomes ( , labeled H2B and H3 respectively) and of the tetrameric particles () are shown. At higher ionic strength (1x PBS) the H2A-H2B dimer is completely released from the octamer when only partial release of the H2A-H2B dimer is observed at low ionic strength (1x TE, 10 mM NaCl).

Fig. 6. Step-wise release of histones at very low chromatin concentrations. **A** - Chromatin aliquots, 4 μg - lines 2 to 6 and 2 μg - lines 7, 8, were diluted in 1xPBS buffer to the indicated concentrations. Positions of the linker histones (H1 and H5) as well as these of the core histones are designated. **B** - Scans of the samples shown in (**A**). Since the amount of loaded material on lanes 7 and 8 was twofold smaller, a normalization of the intensity of the H3 band relative to histone H3 at 20 nM (lane 3) was applied. Note that upon lowering the chromatin concentration the first histones released from chromatin are the linker histones followed by the H2A-H2B dimers.

Fig. 7. Distribution of disruption lengths of reconstituted 5S nucleosmal arrays in presence of nucleosomes at concentration 100 μg/ml. Note the significant increase in the population of disruption with length around 50 nm.

Fig. 8. Stretching profile of the chicken erythrocyte chromatin under optimized conditions (see Materials and Methods). **A** - Force/extension curve (stretching in red, relaxation in black), arrows demark the long ~50 nm disruptions, arrowheads the short ~25 nm ones; **B** - The distribution of disruption lengths. The second well separated maximum at ~50 nm approaches similar statistical weight as the one at ~25 nm.

Table I. Comparison of disruption lengths and threshold forces (±standard deviation) for various chromatin types and experimental conditions. n/a = not applicable.

Chromatin type	**disruption length (nm)**		**average threshold force (pN)**
	1st maximum	2nd maximum	
Native, isolated from chicken erythrocyte nuclei, 100 mM NaCl.	23.2±3.5	40.1±5.5 (shoulder)	19.6±4.6
Native, isolated from chicken erythrocyte nuclei, 50 mM NaCl.	23.2±3.6	51.0±3.3	20.0±7.5
Native, isolated from chicken erythrocyte nuclei, stabilizing conditions, 100 mM NaCl	23.8±4.6	51.7±5.3	20.7±5.0
Reconstituted on 5S rDNA tandem repeat, 100 mM NaCl	24.0±3.3	47.5±5.4	17.6±3.5
Reconstituted on 5S rDNA tandem repeat, 100 mM NaCl, 1 mM $MgCl_2$	23.3±3.4	49.2±4.2	19.1±4.5
Reconstituted on 5S rDNA tandem repeat, 100 mM NaCl, exogenous chromatin at OD=2	25.2±3.9	50.5±3.8	17.8±5.5
H3-H4 tetramer only reconstituted on 5S rDNA tandem repeat , 100 mM NaCl	25.1±4.8	n/a	14.7±1.8

Figure 1

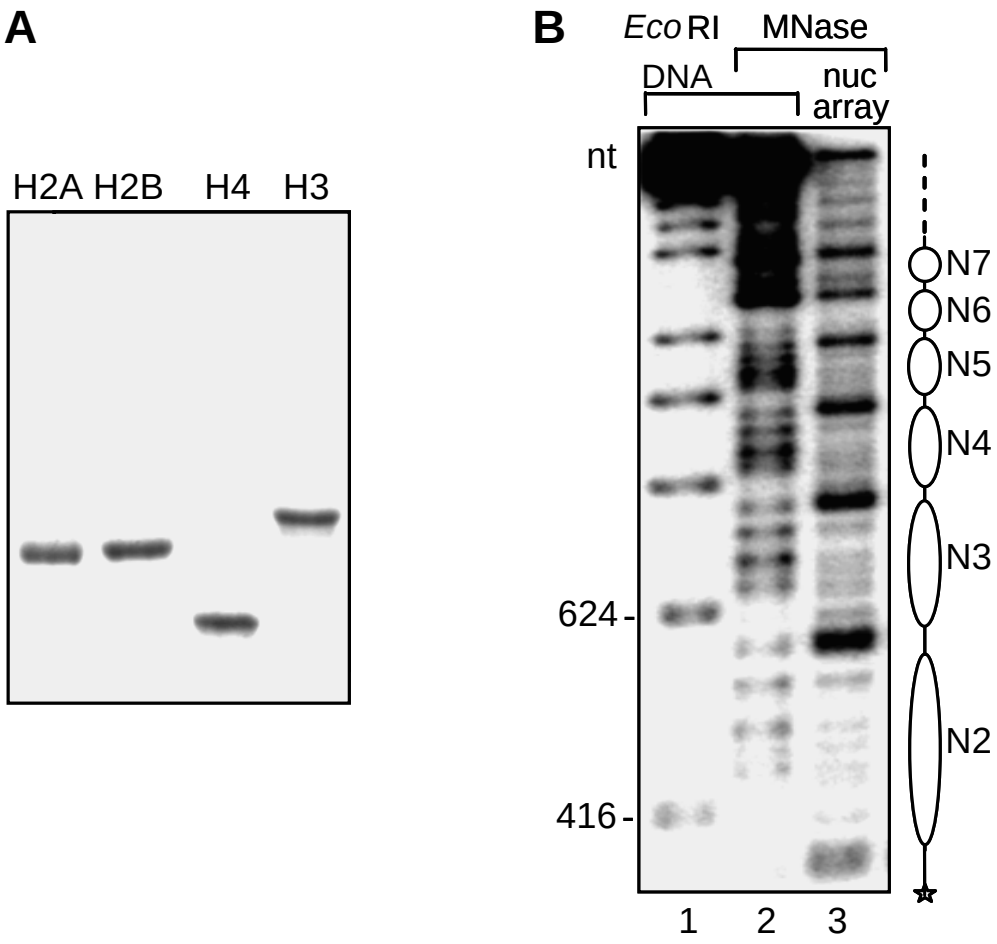

A
H2A H2B H4 H3
B
EcoRI MNase
DNA
nuc array
nt
624
416
N7
N6
N5
N4
N3
N2
1 2 3

Figure 2

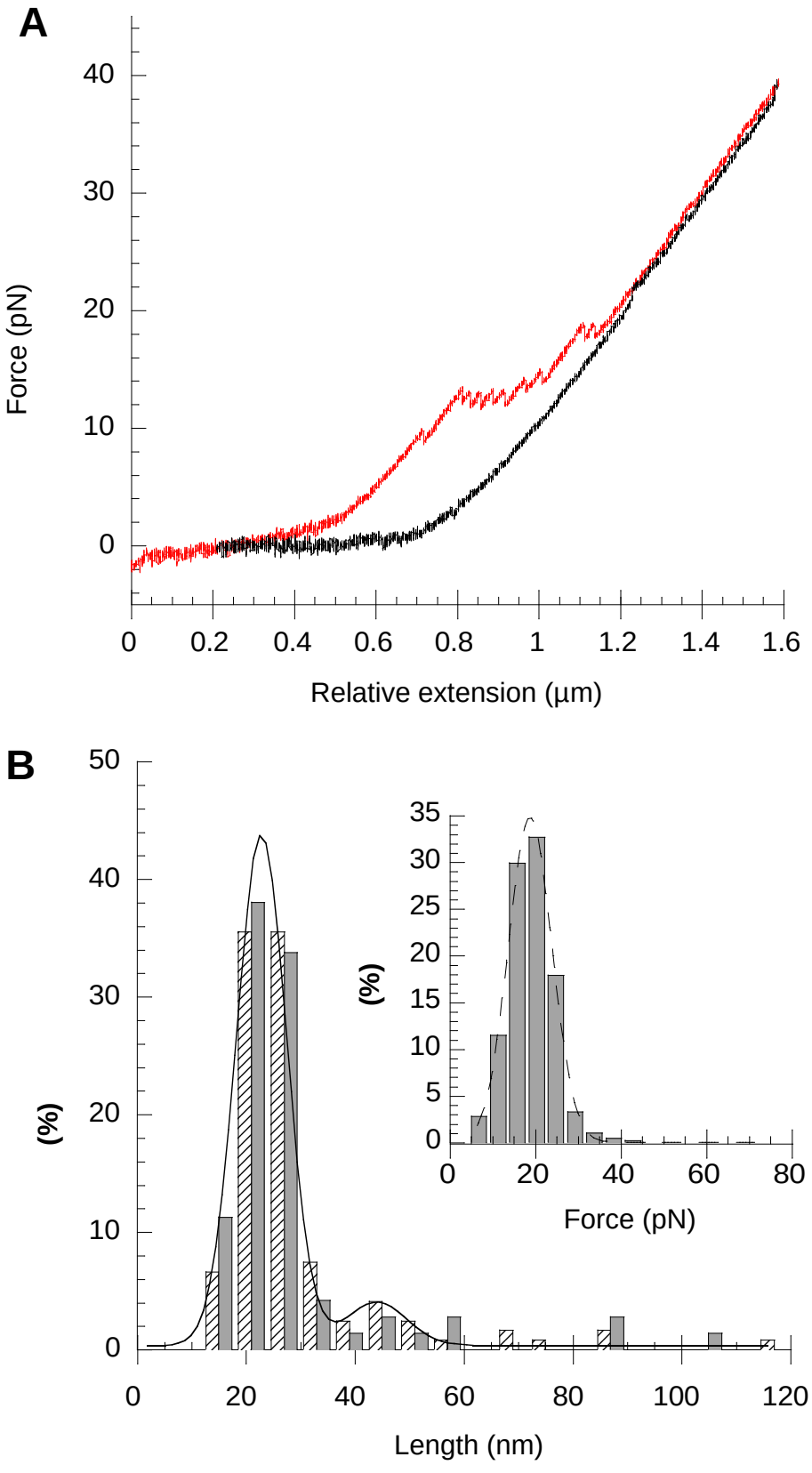

A
Force (pN)
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
Relative extension (µm)
B
(%)
50
40
30
20
10
0
0
20
40
60
80
100
120
Length (nm)
(%)
35
30
25
20
15
10
5
0
0
20
40
60
80
Force (pN)

Figure 3

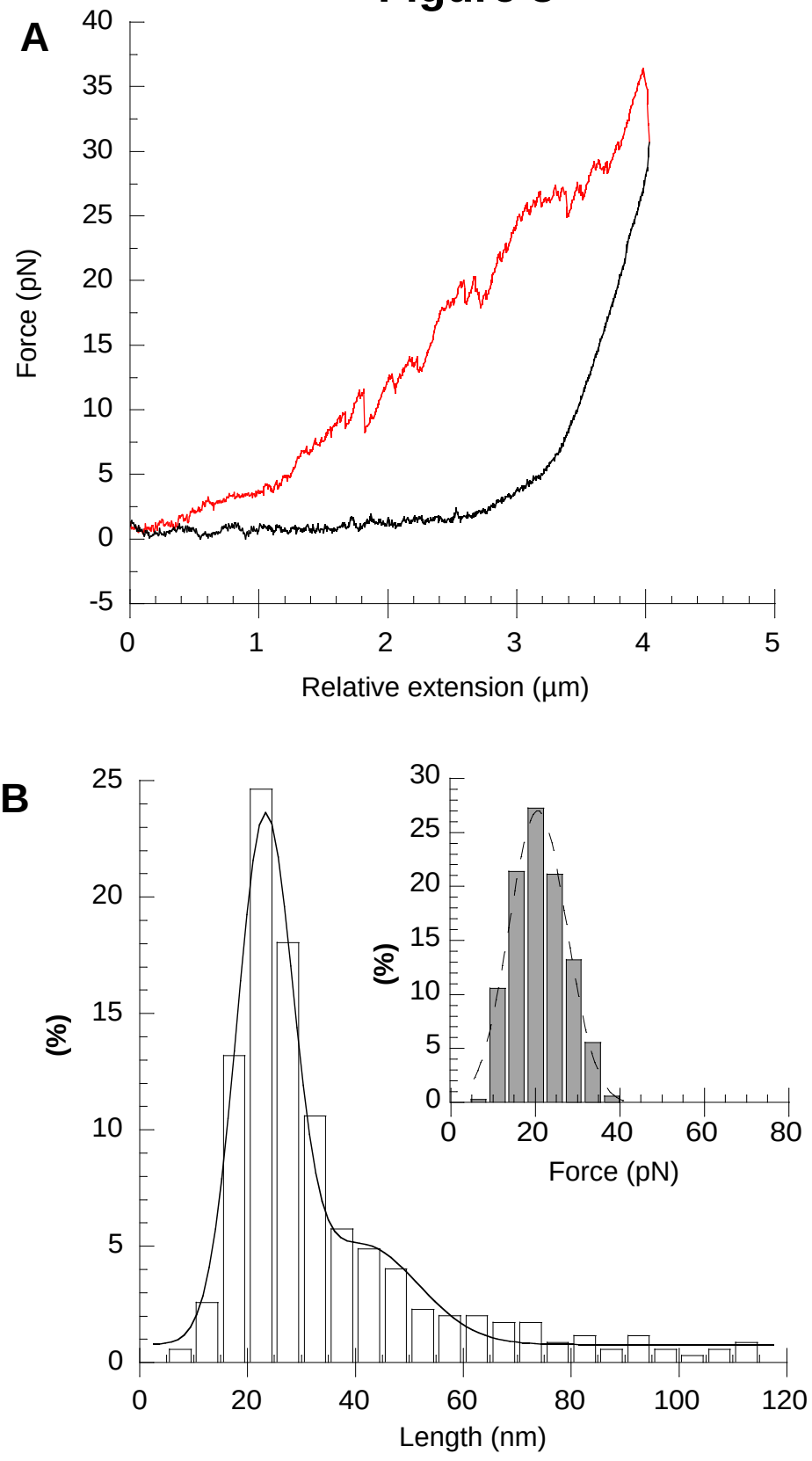


Figure 4

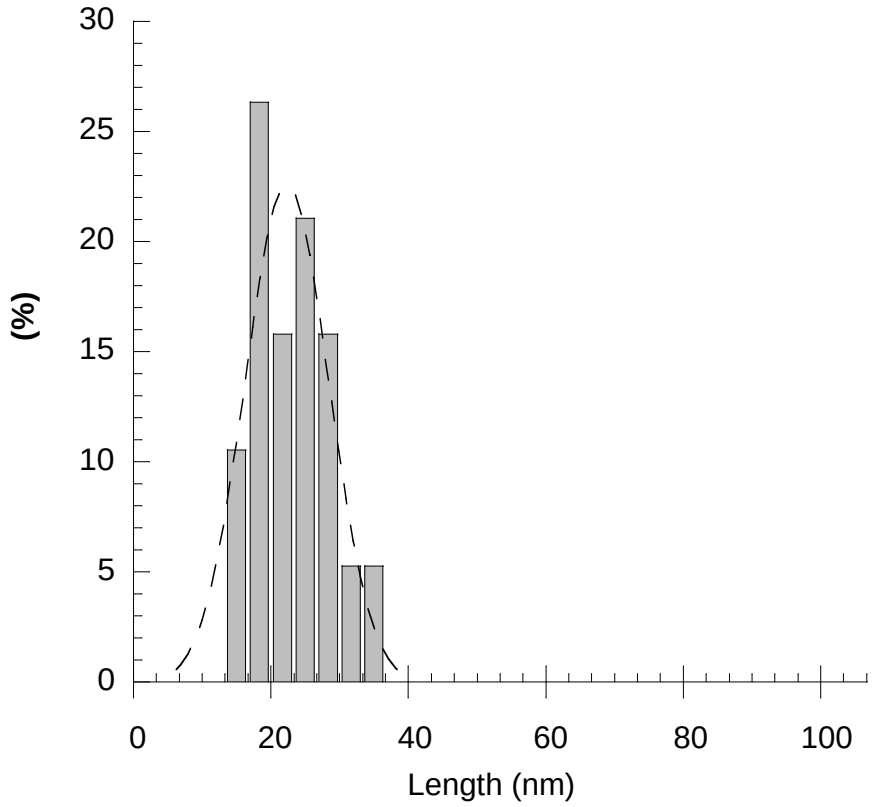


Figure 5

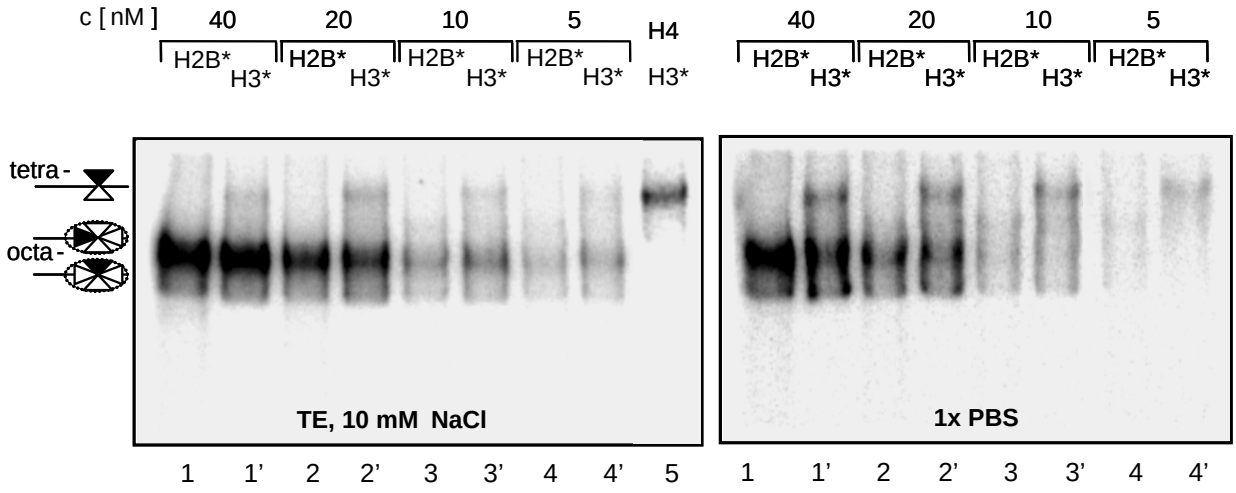

c [nM]
40
20
10
5
H4
H2B*
H3*
H3*
40
20
10
5
H2B*
H3*
tetra -
octa -
TE, 10 mM NaCl
1x PBS
1 1’ 2 2’ 3 3’ 4 4’ 5
1 1’ 2 2’ 3 3’ 4 4’

Figure 6

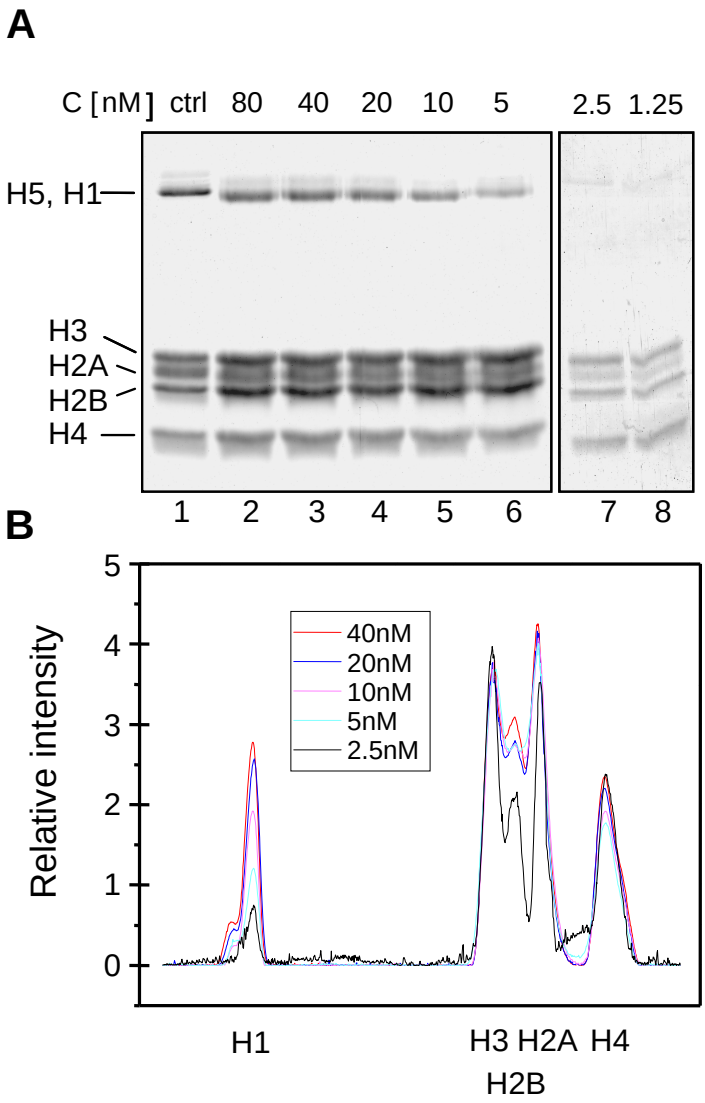

A
C [nM] ctrl 80 40 20 10 5 2.5 1.25
H5, H1
H3
H2A
H2B
H4
1 2 3 4 5 6 7 8
B
Relative intensity
0 1 2 3 4 5
40nM
20nM
10nM
5nM
2.5nM
H1
H3 H2A H4
H2B

Figure 7

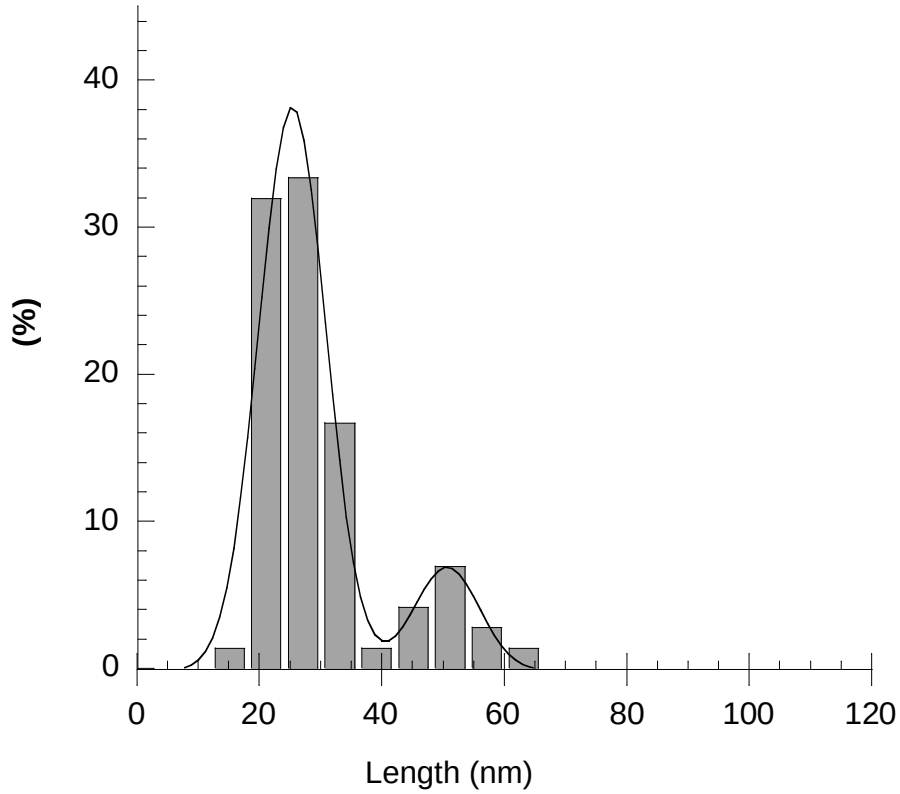
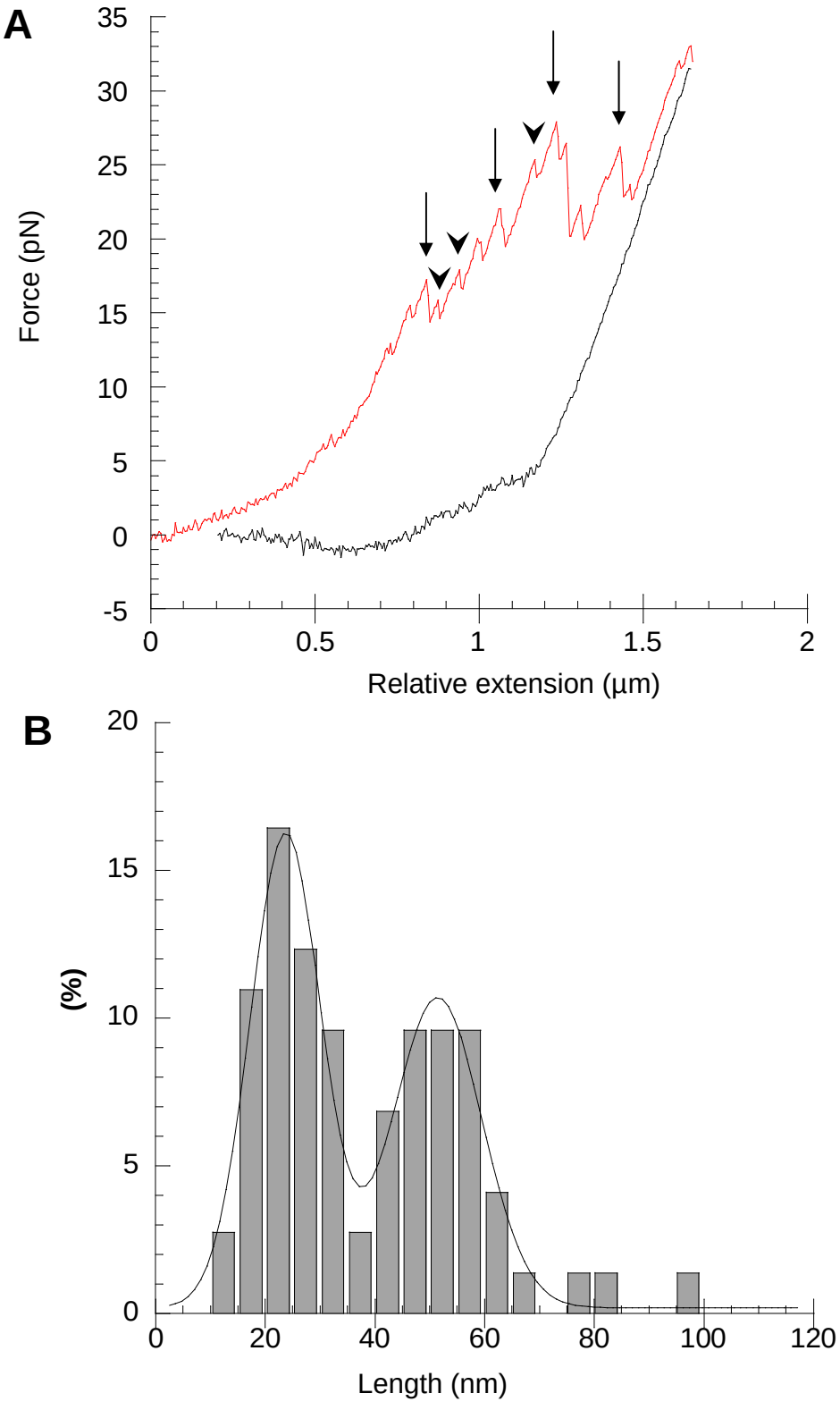


Figure 8


A
35
30
25
20
15
10
5
0
-5
Force (pN)
0
0.5
1
1.5
2
Relative extension (µm)
B
20
15
10
5
0
(%)
0
20
40
60
80
100
120
Length (nm)