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Stability and Reversibility of Higher Ordered Structure of Interphase Chromatin: Continuity of Deoxyribonucleic Acid Is Not Required for Maintenance of Folded Structure[†]

Adolfo Ruiz-Carrillo,* Pere Puigdomènech,[‡] Gabi Eder, and Rudolf Lurz

ABSTRACT: The organization of the higher order structure of chromatin has been examined in chicken erythrocyte. Chromatin solubilized during the time course of a gentle micrococcal nuclease digestion of nuclei shows a continuous variation in the distribution of molecular weights. Electron microscopy studies of large chromatin fragments solubilized at physiological ionic strength (0.14 M NaCl or KCl) suggest that the polynucleosome chain is folded in continuous compact structures of an average diameter of 23 nm in which the individual nucleosomes are difficult to distinguish. This compact structure is destabilized even at intermediate ionic strengths (e.g., 40 mM NaCl), resulting in looser fibers of similar diameter. At 5 mM NaCl the fiber is unraveled into a continuous filament of 10-nm diameter. These conformational

changes are reversible as determined by hydrodynamic and biochemical parameters. The 10-nm → 23-nm transition of chromatin appears to be a cooperative process requiring the full complement of histones H1 and H5. Micrococcal nuclease cleaves the DNA in the compact chromatin structure to an apparent limit of digestion corresponding to an average of eight to nine nucleosomes with little effect on the size of the fiber. Thus, the continuity of the DNA is not required for the stability of the folded chromatin fiber. Histones H1 and H5 exhibit a binding preference to larger chromatin fragments regardless of the length of the DNA. This behavior is not observed with relaxed chromatin, suggesting that multiple stabilizing interactions involving H1 (H5) are possible only in the compact configuration.

The DNA of the somatic cells of higher organisms is packaged inside the nucleus through its association with nuclear proteins among which histones are known to play a major structural role. The primary reduction in the apparent length of DNA occurs in the chromatin subunit called the "ν body" (Olins & Olins, 1974) or nucleosome (Oudet et al., 1975). The nucleosome, in its compact configuration, affords a DNA packing ratio of ~7:1 (Sperling & Tardieu, 1976). An extended chain of closely packed nucleosomes occurring in solutions of low ionic strength can be correlated with the 10-nm fiber or nucleofilament (Finch & Klug, 1976). Since the packing ratio estimated for the bulk of the interphase chromatin is ~100:1 (Beerman, 1972), further condensation of the polynucleosome chain is necessary. Numerous laboratories have studied interphase chromatin by electron microscopy, and it is now widely accepted that when suitable ionic conditions are maintained chromatin appears as fibers having a heterogeneous diameter varying between 20 and 30 nm (Ris & Kubai, 1970; Davies, 1968; Finch & Klug, 1976; Brasch, 1976)

(which we will refer to as the 25-nm fiber). Therefore, it is reasonable to infer that the 25-nm fiber may represent the native state of the majority of the (nonactive?) interphase chromatin, although higher orders of organization cannot be excluded.

Two models for the structure of the 25-nm fiber have been proposed. In the solenoid model (Finch & Klug, 1976), the 10-nm fiber would represent the least coiled member of a family of superhelical chromatin structures in which the 25-nm fiber would be the next hierarchical member. A more recent model (Renz et al., 1977; Hozier et al., 1977) proposes that the polynucleosome chain folds discontinuously into globular structures termed "superbeads" containing 6-10 nucleosomes. Regardless of the actual structure of the 25-nm fiber, a number of studies implicate H1 in the organization of the superstructure of chromatin (Littau et al., 1965; Finch & Klug, 1976; Renz et al., 1977; Thoma & Koller, 1977). However, results also suggesting a solenoid model have been obtained by neutron scattering studies of chromatin at high concentration in the absence of histone H1 (Carpenter et al., 1976). Studies on the role of H1 in the structure of chromatin have been limited by the insolubility of H1-containing chromatin at or near to physiological ionic strength [see, for instance, Oth & Desveux (1957) and Bradbury et al. (1973)]. In most studies chromatin has been extracted and handled in low or

[†]From the Max-Planck-Institut für Molekulare Genetik, Berlin-Dahlem, 1000 Berlin 33, Federal Republic of Germany. Received November 14, 1979.

[‡]Dr. Puigdomènech is a current member of the Institut de Biologia Fonamental, Departament de Bioquímica (Ciències), Universitat Autònoma de Barcelona, Barcelona, Spain.

intermediate ionic strength buffers (i.e., 0.2 mM EDTA¹ to 80 mM NaCl). Since it is unknown to what extent the transition between compact and relaxed chromatin is reversible, the results obtained with soluble chromatin are difficult to interpret.

In this communication we report detailed studies on the properties of large chromatin fragments solubilized at 40–140 mM NaCl or KCl after controlled micrococcal nuclease digestion of nuclei. We describe some aspects of the folding, organization, and maintenance of the 25-nm chromatin fiber and the involvement of histones H1 and H5 in this process.

Experimental Procedures

All operations were carried out between 2 and 4 °C unless specified otherwise.

Preparation of Cells and Nuclei. Mature chicken erythrocytes were obtained as described previously (Ruiz-Carrillo et al., 1975). Nonerythroid blood cells were removed by absorption to sulfopropyl-Sephadex (Pharmacia) columns (Perucho et al., 1979). Cells were lysed with 0.5% (v/v) Nonidet P40 either in buffer A (90 mM KCl, 30 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 0.4 mM phenylmethanesulfonyl fluoride (PMSF), and 10 mM triethanolamine hydrochloride, pH 7.3) or in buffer B (110 mM KCl, 30 mM NaCl, 0.4 mM PMSF, 0.2 mM MgCl₂, and 10 mM triethanolamine hydrochloride, pH 7.3). Nuclei were washed 4 times in the corresponding buffer containing 0.5% Nonidet P40 and finally in buffer A or B.

Preparation of Soluble Chromatin. Two different procedures for the preparation of soluble chromatin were followed.

(1) **Solubilization at 40 mM NaCl.** Nuclei isolated in buffer A were resuspended in the same buffer containing 1 mM CaCl₂ to a DNA concentration between 4 and 6 mg/mL and were digested with 60 units of micrococcal nuclease (Worthington) per mg of DNA unless otherwise indicated. The reaction was stopped by the addition of sodium ethylenediaminetetraacetate (EDTA) to 2.5 mM. The amount of acid-soluble DNA was determined as described (Jorcano & Ruiz-Carrillo, 1979). After digestion, nuclei were gently pelleted and resuspended in buffer C (1 mM EDTA, 10 mM triethanolamine hydrochloride, and 0.4 mM PMSF, pH 7.3) containing 0.14 M NaCl and diluted slowly with 3.5 volumes of buffer C up to 40 mM NaCl at the original DNA concentration. After extraction for 1–12 h, the nuclear lysates were centrifuged at 3000 rpm and the pellets were reextracted twice as described above.

(2) **Solubilization at 140 mM Salt.** Nuclei isolated in buffer B were digested as indicated in procedure 1. Digested nuclei were gently shaken overnight, and the solubilized chromatin was recovered in the supernatant after centrifugation at 2000 rpm for 10 min. The pellets were reextracted in 140 mM KCl in buffer C twice by the same procedure.

Soluble chromatin was dialyzed 24 h against buffer D (10 mM triethanolamine hydrochloride, 0.4 mM PMSF, and 0.2 mM EDTA, pH 7.3) with the appropriate salt concentration. In the experiments in which the ionic strength was increased, the salt molarity was raised either in 10 mM NaCl steps,

~50–70 min apart, or continuously at a rate of 6.8 mM/h. Where indicated, chromatin was treated with RNase A (50 µg/mL) at 37 °C for 30 min.

Removal of H1 and H5. Soluble chromatin was depleted of histones H1 and H5 according to a modification of the method of Bolund & Johns (1973). Chromatin was made 0.35 M in NaCl by the quick addition of 0.66 M NaCl in buffer D while vortexing. The clear solution of chromatin (1.4 mg/mL) was then added to 0.65 volume of AG 50W-X2 (Bio-Rad) equilibrated with the same buffer and left with gentle agitation for 2.5 h at room temperature. After centrifugation, chromatin was then dialyzed 24 h against the desired salt concentration in buffer D. After this treatment, H1 and H5 were removed from chromatin without depletion of the core histones as determined by gel electrophoresis and sedimentation analysis of the resulting chromatin. The repeat length of H1,H5-depleted chromatin was similar to that of control chromatin as determined by electrophoretic analysis of the DNA fragments produced by micrococcal nuclease (unpublished observations).

Digestion of Soluble Chromatin. Soluble chromatin or H1,H5-depleted chromatin at the specified salt molarity in buffer D was made 0.6 mM in CaCl₂ (final effective concentration 0.2 mM CaCl₂) and further digested with micrococcal nuclease (60 units of enzyme per mg of DNA). The reaction was stopped by raising the EDTA concentration to 0.6 mM.

Soluble chromatin was digested with trypsin (Serva, TPCK treated, 40 units/mg) at a ratio of 1 µg of enzyme per 1000 µg of DNA. The reaction was stopped by addition of soybean trypsin inhibitor (Serva) (3 µg of inhibitor per µg of trypsin).

Glutaraldehyde Cross-Linking. Soluble chromatin or H1,H5-depleted chromatin at the desired ionic strength in buffer D was reacted with glutaraldehyde (Fluka, purissimum). Only glutaraldehyde solutions which had an A₂₃₀/A₂₈₀ ratio smaller or equal to 0.4 were used (Anderson, 1967). Typically, chromatin solutions at a DNA concentration of 1.3 mg/mL were reacted with 0.2% (w/v) glutaraldehyde at 0 °C. The reaction was either stopped by adding trichloroacetic acid to 18% (w/v) or quenched by adding 1.5 volumes of 0.4 M Tris-HCl, pH 8.0. In some experiments H1 and H5 were selectively extracted by stopping the reaction with 5% (w/v) perchloric acid. After the pellets were washed with 5% perchloric acid, the pellet and supernatant fractions were made 18% in trichloroacetic acid and prepared for electrophoresis as described by Ruiz-Carrillo & Jorcano (1979).

Analytical Methods. Samples of chromatin were sedimented through 5–20% sucrose linear gradients in buffer D containing NaCl or KCl at the same concentration of the loaded samples. When samples of chromatin were pretreated with trypsin, soybean trypsin inhibitor at 1 µg/mL was included in all solutions. Centrifugations were carried out in a Beckman SW40 Ti rotor at 39 000 rpm for 80–150 min. Gradients were monitored at 254 nm. Apparent sedimentation coefficients of chromatin were calculated relative to those of immature chicken erythrocyte polysomes (Perucho et al., 1979) run in parallel gradients.

Proteins were analyzed in 15–19% NaDodSO₄-polyacrylamide gradient slab gels as described previously (Ruiz-Carrillo & Jorcano, 1979). Quantitative estimation of the histones was carried out by scanning the gel in a Vitatron microdensitometer equipped with a Spectra-Physics integrator, as described previously (Ruiz-Carrillo & Jorcano, 1979). Sonicated erythrocyte nuclei at similar protein concentrations to those of the test samples were included in all electrophoreses, and

¹ Abbreviations used: buffer A, 90 mM KCl, 30 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 0.4 mM PMSF, and 10 mM triethanolamine hydrochloride, pH 7.3; buffer B, 110 mM KCl, 30 mM NaCl, 0.2 mM MgCl₂, 0.4 mM PMSF, and 10 mM triethanolamine hydrochloride, pH 7.3; buffer C, 1 mM EDTA, 0.4 mM PMSF, and 10 mM triethanolamine hydrochloride, pH 7.3; buffer D, 0.2 mM EDTA, 0.4 mM PMSF, and 10 mM triethanolamine hydrochloride, pH 7.3; PMSF, phenylmethanesulfonyl fluoride; EDTA, sodium ethylenediaminetetraacetate; NaDodSO₄, sodium dodecyl sulfate.

the relative areas of the histone bands were taken as reference.

DNA was purified by deproteinization with 0.1 mg/mL proteinase K (Merck) in 0.1% NaDodSO₄, 200 mM NaCl, 0.2 mM EDTA, and 10 mM triethanolamine hydrochloride (pH 7.3) for 4–48 h at 37 °C and phenol–chloroform (1:1) extraction. The DNA was immediately precipitated by addition of 2 volumes of ethanol. For DNA of high molecular weight the precipitation step was omitted and samples were directly analyzed in gels. DNA was analyzed in vertical agarose (Seakem, ME) slab gels, the porosity of which was varied between 0.7 and 2%, run at 1–5 V/cm. High molecular weight DNA was analyzed in vertical 0.25–0.3% agarose [Seakem, HGT (P)] slab gels run at 0.1–0.2 V/cm (Fangman, 1978). Electrophoresis buffer was in all cases 35 mM Tris, 30 mM KH₂PO₄, and 1 mM EDTA (pH 7.9). Gels were stained and photographed as described (Jorcano & Ruiz-Carrillo, 1979), and the negatives were scanned with a Joyce-Loebl microdensitometer. The lengths of the DNA were estimated against the mobility of the DNA from the following markers: P1, λ , λ restricted with endoR *Hind*III (Miles) (Maniatis et al., 1975), and λ dv1 restricted with endoR *Bsu* (Steinmetz et al., 1978).

Electron Microscopy. Chromatin samples at 50 μ g/mL were either not fixed or fixed with 0.1% glutaraldehyde (Fluka, purissimum) for 18 h at 0 °C. The efficiency of fixation was monitored by electrophoretic analysis of the histones (Ruiz-Carrillo & Jorcano, 1979).

Negative staining was performed as described by Valentine et al. (1968) with 2% uranyl formate, pH 4.5. Carbon films were picked up with neoprene-treated 400-mesh copper grids. Grids were examined with an EM 301 electron microscope (Philips, Eindhoven) at an acceleration voltage of 80 kV at calibrated primary magnifications of 57 000.

Results

Analysis of the Size of Chromatin Solubilized by Micrococcal Nuclease. To study the role of H1 (H5) in the organization of chromatin, we have used micrococcal nuclease digestion to reveal higher order structural features of interphase chromatin. This approach is likely to reflect the behavior of the bulk of chromatin which in the mature erythrocyte is very inactive.

Experimental conditions were optimized so that a relatively synchronous digestion of all nuclei could be achieved; two methods to solubilize fragmented chromatin were used (see Experimental Procedures). Figure 1A shows the time course of hen erythrocyte nuclei digestion by micrococcal nuclease at 4 °C. Under the conditions used, only minimal amounts of UV-absorbing material were released, while the majority of the chromatin remained inside the nucleus. The released material contained small DNA fragments which were acid soluble and a low percentage of relatively small nucleosome oligomers which were converted very quickly to nucleosome monomers. This material had the full complement of the core histones but was depleted of histones H1 and H5 (results not shown). To solubilize the bulk of fragmented chromatin from nuclei digested in buffer A, we decreased the ionic strength of the medium to 40–60 mM NaCl (procedure 1). Solubilization of chromatin has a lag period during the first 10 min of nuclease digestion. After this lag, the amount of chromatin solubilized increased very rapidly even though the size of the DNA is still relatively large. It appears that a minimum number of cuts on the DNA are required to release chromatin in which the DNA length can exceed 10⁵ base pairs at the earlier times (Figure 1A, a and b of insert). This is in agreement with recent observations in which different tissue

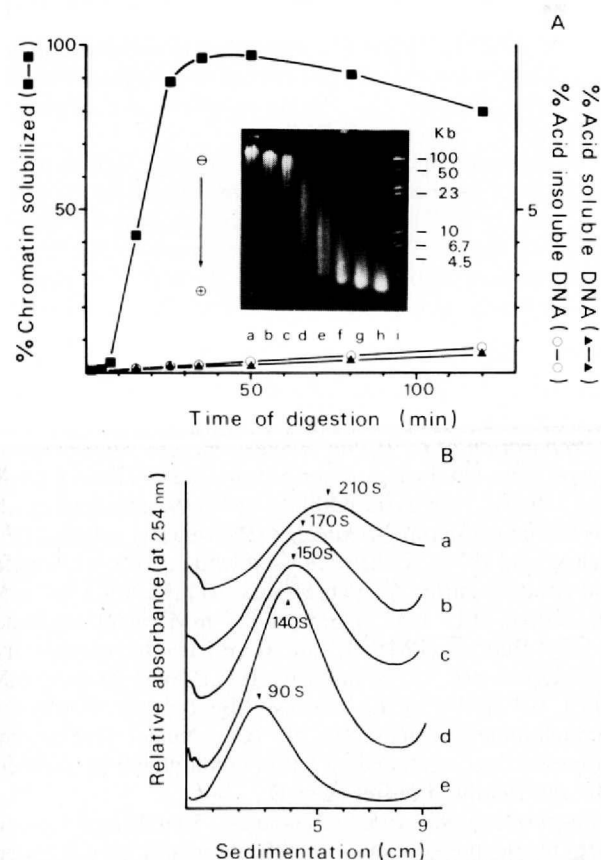


FIGURE 1: Time course of chromatin digestion by micrococcal nuclease. Hen erythrocyte nuclei were digested at 3 to 4 °C with micrococcal nuclease. At different times during the digestion aliquots were withdrawn and the reaction was stopped by the addition of EDTA to 2.5 mM. Digested nuclei were pelleted, and the amount of DNA solubilized was determined in the supernatant. Chromatin was solubilized in 40 mM NaCl according to procedure 1 (see Experimental Procedures). (A) Percentage of acid-soluble DNA (▲) and acid-insoluble DNA (○) in the first supernatant after the digestion with 60 units of micrococcal nuclease per mg of DNA. (■) Cumulative percentage of chromatin solubilized at 40 mM NaCl in three consecutive extractions. Inset: 0.3% agarose gel electrophoresis of total DNA at different times during the digestion. (a–h) 3, 5, 7, 15, 25, 50, 80, and 120 min; (i) a mixture of DNA from P1, λ , and endoR *Hind*III restriction fragments of λ . The molecular weight of the standards is given in kilobases (1 kb = 1000 base pairs). (B) Sucrose gradient sedimentation analysis of chromatin extracted by procedure 1 after (a–e) 5, 7, 10, 15, and 38 min of digestion with 30 units of micrococcal nuclease per mg of DNA. Solutions contained 40 mM NaCl in buffer D. Sedimentation was for 50 min.

and digestion conditions were used (Igo-Kemenes & Zachau, 1977). After obtaining close to 100% solubilization, the yield of extracted chromatin decreased as the digestion proceeded. This may be related to a precipitation of chromatin caused by the accumulation of H1 and H5 (see below, Figure 7 and Table I). Chromatin solubilized at 40 mM NaCl during the first hour of the digestion appears to have the full complement of all histones as determined by gel electrophoresis (not shown). Analysis of the chromatin fragments by sucrose gradient centrifugation showed a decrease in the *s* value as digestion progressed (Figure 1B). When digestion was allowed to proceed even further, the chromatin fragments became smaller until the familiar peaks of nucleosome monomers and oligomers were resolved in the distribution (results not shown). As expected, these results were independent of RNase A pretreatment of the chromatin (Figure 1B) since the RNA content of the mature erythrocyte nuclei is vanishingly small (Ruiz-Carrillo et al., 1974). DNA extracted from chromatin during the course of the digestion also showed a continuous

Table I: Accumulation of H1 and H5 in DNA-Cleaved Folded Chromatin Fibers during Micrococcal Nuclease Digestion

material	digestion time (min) ^a	% chromatin pptd ^b	(H1 + H5)/core histones ^c	
			ppt	total supernatant
nuclei	0		0.30	
soluble chromatin ^d	0		0.29	
peak gradient ^e	0		0.34	
chromatin	10	14	0.30	0.28
peak gradient	10		0.36	
chromatin	25	25	0.31	0.27
peak gradient	25		0.37	
chromatin	40	33	0.32	0.26
peak gradient	40		0.41	
chromatin	60	41	0.34	0.25
peak gradient	60		ND ^f	
chromatin	80	44	0.36	0.24
peak gradient	80		0.43	

^a Micrococcal nuclease digestion (0.06 unit/ μ g of DNA) was carried out at 4 °C in the presence of 0.6 mM CaCl₂. ^b Extent of chromatin precipitation during the digestion as determined after centrifugation at 12 000 rpm for 2 min at 4 °C. ^c Ratio determined by densitometric analysis of the histones separated by electrophoresis. ^d Refers to the same experiment shown in Figure 5. ^e Corresponds to the chromatin sedimenting with the constant *s* value (shadowed) in the gradients shown in Figure 5A. ^f Not determined.

distribution in molecular weights (Figure 1A, inset). These data are compatible with a random cleavage of the DNA in chromatin at many susceptible points.

Structural Transitions of Chromatin. To gain information on the salt-dependent folding of chromatin, we studied its hydrodynamic properties in the presence of several concentrations of monovalent ions. Transitions in the conformation of chromatin result in changes in sedimentation velocity as determined by centrifugation in sucrose gradients containing 5, 40, and 100 mM NaCl. These results are summarized in Figure 2A, in which the average nucleosome number is plotted as a function of the average sedimentation coefficient (*s*). The polynucleosome fiber experiences a relaxation at the lowest (plot c) and a compaction at the highest (plot a) ionic strength studied. The *s* values are proportional to the molecular weight (M_r) of the fiber following the relationship $M_r^{0.61}$ (5 mM NaCl), $M_r^{0.71}$ (40 mM NaCl), and $M_r^{0.74}$ (100 mM NaCl), the increase in the exponent being an indication of a less extended conformation (Tanford, 1961).

To study the effect of H1 and H5 in the salt-dependent structural transitions, we selectively removed these histones from soluble chromatin by treatment with the ionic exchanger AG 50W-X2 (Bolund & Johns, 1973). The results shown in Figure 2B indicate that at 100 mM NaCl (or KCl) H1,H5-depleted chromatin (b) is more relaxed than H1,H5-containing chromatin (a). This increase in flexibility is comparable to the unfolding experienced by the H1,H5-containing chromatin at 5 mM NaCl (Figure 3d) and at 0 mM NaCl (Figure 2B, c). The levels of unfolding of depleted chromatin at 0 mM NaCl (Figure 2B, d) are much higher than those in control chromatin (c). In fact, the *s* values of H1,H5-depleted chromatin under these conditions are very close to those of the corresponding naked DNA (not shown). This suggests that in the absence of H1 and H5 further levels of disorganization may occur, possibly due to the unfolding of the core particle, as shown for isolated core particles (Gordon et al., 1978).

To correlate the hydrodynamic behavior of chromatin with morphological features, we have examined chromatin at different ionic strengths by electron microscopy. For these studies, large chromatin fragments were obtained in a state

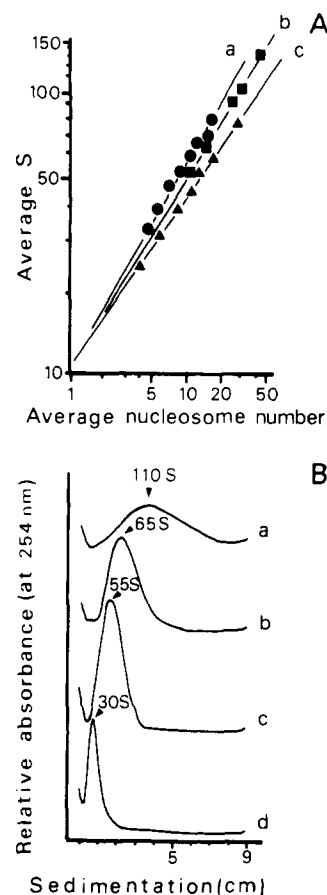


FIGURE 2: Salt-dependent structural transitions of chromatin fibers. Effect of H1 and H5 removal. (A) Chromatin extracted from nuclei according to procedure 1 after 10 min of micrococcal nuclease digestion was dialyzed against 5, 40, and 100 mM NaCl in buffer D and analyzed by sucrose gradient centrifugation. The average *s* value was calculated from the distance sedimented by the chromatin at the midpoint of a fraction (0.6 mL). The average number of nucleosomes of chromatin was calculated from the weight-average molecular weight of the DNA of a corresponding fraction. (This was determined by planimetry of densitometric tracings of the DNA electrophoresed in 0.7% agarose slab gels.) The plot shows the dependence of the average *s* value of chromatin on the molecular weight at (a–c) 100, 40, and 5 mM NaCl, respectively. (B) Chromatin extracted as in (A) was depleted of H1 and H5 by treatment with AG 50W-X2. After depletion, chromatin was dialyzed 24 h against 100 or 0 mM NaCl in buffer D. Control chromatin was simply dialyzed against 100 or 0 mM NaCl in buffer D. H1,H5-depleted (b and d) and control (a and c) chromatin samples were sedimented in sucrose gradients for 70 min. (a and b) 100 mM NaCl; (c and d) 0 mM NaCl.

as close as possible to its native configuration after very brief micrococcal nuclease digestion of nuclei. It was found that removal of polyamines from the buffer together with longer extraction times permitted the solubilization of chromatin containing intact histones (Figure 9D, a) at physiological ionic strength (i.e., 0.14 M monovalent ions, procedure 2). Chromatin extracted in this manner will henceforth be referred to as folded chromatin. Figure 3 shows electron micrographs of chromatin of an average length of 500 nucleosomes. At 140 mM NaCl, chromatin appears as a compact fiberlike structure rather homogeneous in contour, with an average diameter of ~23 nm. Under these conditions some fibers show a clear tendency to coil into even higher order structures ~80 nm in diameter (arrow heads). Nucleosomes are very difficult to distinguish in the very compact stretches, suggesting that they may be closely packed. A superhelical arrangement of nucleosomes can be distinguished in some sections of the fiber (arrows) and in small chromatin fragments such as the one

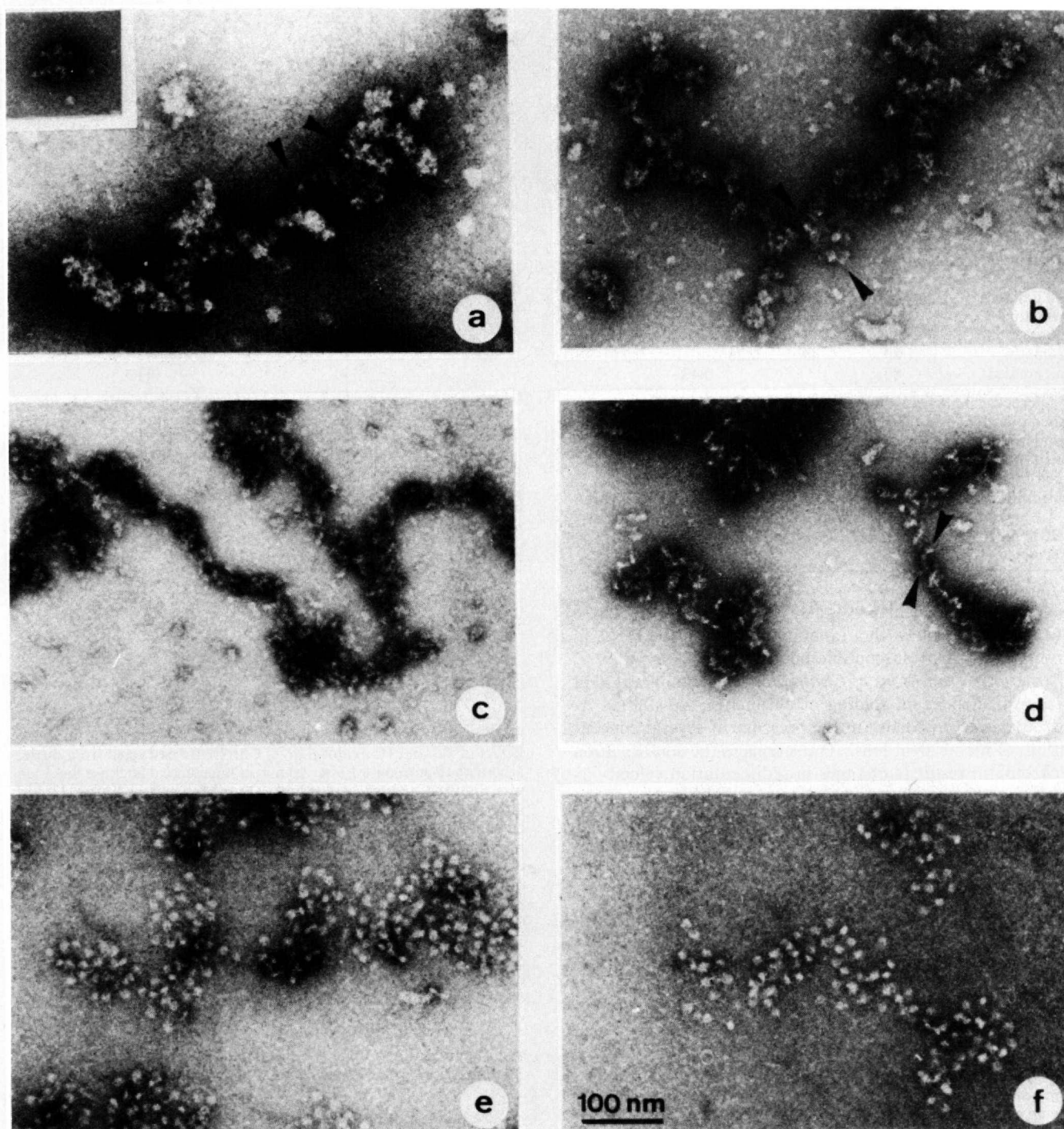


FIGURE 3: Visualization of structural transitions of chromatin fibers. Chromatin was extracted at physiological ionic strength according to procedure 2 (see Experimental Procedures) from nuclei digested with micrococcal nuclease for 2 min and dialyzed against 140, 100, 40, and 5 mM KCl in buffer D containing 5 mM triethanolamine hydrochloride. Chromatin was fixed with 0.1% glutaraldehyde and negatively stained with 2% uranyl formate, pH 4.5. (a-d) Chromatin fibers at 140, 100, 40, and 5 mM KCl, respectively. Arrow heads point to right-handed secondary coiling of the primary fibers. The arrows in (a) point to a region of the fiber in which nucleosomes can be seen. The inset in (a) shows a chromatin fragment in which nucleosomes appear to be in a helical configuration. (e and f) Unfixed chromatin fibers in 100 and 40 mM KCl, respectively.

shown in the inset of Figure 3a. At 100 mM NaCl (b) the fiber has characteristics similar to the 140 mM fiber, also with an average diameter of ~ 23 nm, although its degree of compactness may be slightly lower as determined by a decrease in the sedimentation coefficient. A clear relaxation of the fiber can be observed at 40 mM NaCl (c). However, the average diameter was still around 23 nm, suggesting that the fiber is less compact but not yet unraveled. At 5 mM NaCl (d) chromatin was seen as a continuous filament of ~ 10 -nm diameter, indicating that the 23-nm fiber is fully unraveled.

At this low ionic strength or even at 0 mM NaCl (only buffer D, not shown), "beads-on-a-string" nucleosomes were never observed. The morphology of the 10-nm fiber suggests that under our conditions of specimen preparation, the nucleosome "linker" region is not in an extended conformation. These morphological transitions were only observed in chromatin cross-linked with glutaraldehyde or glutaraldehyde and formaldehyde. When fixation was omitted, beads-on-a-string nucleosomes were observed independent of the ionic strength (Figure 3, e and f).

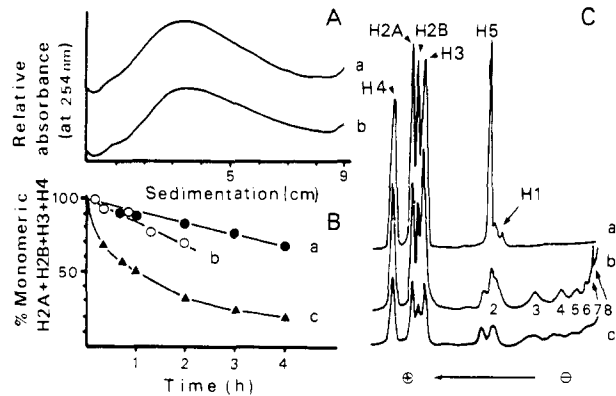


FIGURE 4: Effect of H1 and H5 on the rate of glutaraldehyde cross-linking of histones in chromatin. Chromatin solubilized according to procedure 1 after a 15-min micrococcal nuclease digestion of nuclei was depleted of H1 and H5 and dialyzed against 100 mM NaCl in buffer D containing 5 mM triethanolamine hydrochloride. Nontreated chromatin was dialyzed against the same buffer solutions containing either 100 or 0 mM NaCl. Glutaraldehyde was added to a final concentration of 0.2%, and the reaction was either not quenched (A) or quenched (B and C) at the indicated times. (A) Sedimentation profiles of equal amounts of control chromatin at 100 mM NaCl (a) or after 15 h of cross-linking with glutaraldehyde (b). Samples were centrifuged for 70 min. (B) Rate of cross-linking of core histones. Equal amounts of quenched cross-linked H1,H5-containing chromatin at 0 (●) and 100 mM NaCl (▲) and of H1,H5-depleted chromatin at 100 mM NaCl (○) were analyzed by electrophoresis. Quantitative estimation of the histones on the gel was carried out by densitometry. (C) Densitometric tracings of the electrophoretic separation of histones from H1,H5-containing chromatin: (a) non-cross-linked chromatin; (b) chromatin at 0 mM NaCl reacted for 4 h; (c) chromatin at 100 mM NaCl reacted for 2 h. The numbers in (b) refer to the mobility of the core histone multimers.

The condensation of the chromatin fibers observed with an increase of ionic strength (Figures 2A and 3) suggested that the distance among nucleosomes might change during the structural transition. This was determined by the use of glutaraldehyde which at neutral pH reacts with the free amino groups of histones, producing oligomers and polymers (Olins & Wright, 1973). Control experiments indicated that glutaraldehyde does not detectably cross-link histones to DNA as determined by electrophoresis of DNA extracted from cross-linked and control chromatin (results not shown). Therefore, the reaction of glutaraldehyde with chromatin can be used as a probe of histone proximity. Figure 4B shows that the rate at which the monomeric core histones are converted into oligomers and polymers is three- to fourfold higher in soluble chromatin fibers at 100 mM (c) than at 0 mM (a) NaCl. H1 and H5 were also cross-linked at a higher rate at 100 mM than at 0 mM NaCl as determined by selective extraction with 5% perchloric acid (Johns, 1964). The faster cross-linking rate observed at 100 mM NaCl is not only the result of unspecific condensation of the polynucleosome fiber due to shielding of the DNA phosphate by salt since at the same ionic strength H1,H5-depleted chromatin showed a decrease in the rate of cross-linking (b). Electrophoretic analysis of the histones (Figure 4C) indicated that the cross-linked products follow the progression from dimers to oligomers larger than eight that do not enter the gel. This suggests that glutaraldehyde is cross-linking the histone not only inside individual nucleosomes but also among neighboring subunits. Thus, at 100 mM NaCl the nucleosomes are closer together than at 0 mM NaCl.

The closer proximity of the nucleosomes at 100 mM NaCl could be due either to their packing in individual fibers or to a side-by-side aggregation among polynucleosome chains. Figure 4A shows that the distribution of s values of control

and glutaraldehyde cross-linked chromatin at 100 mM NaCl is virtually identical. In the outset, the similarity between both sedimentation profiles could be interpreted to mean that nucleosomes become cross-linked either in individual fibers or by unspecific side-by-side aggregation among polynucleosomal chains. The latter would be the case if the sedimentation profile of control chromatin reflected the noncovalent interaction of side-by-side aggregates which would simply be covalently linked as a result of glutaraldehyde treatment. In this case one would expect to see heterogeneous DNA lengths in soluble chromatin fractionated according to size but this was not observed (Figure 2A). In addition, electron microscopy analysis of cross-linked chromatin failed to detect the presence of large aggregates or fibers with big variations in diameter. Although we cannot exclude the possibility that some interfiber cross-linking may occur, these results are best interpreted as a reflection of the closer packing of nucleosomes in the 23-nm fiber. Independent lines of evidence, such as the light scattering studies of chromatin fibers (Campbell et al., 1978), support our interpretation.

Properties of the Higher Order Structure of Chromatin.

The slow release of chromatin from nuclease-digested nuclei at physiological ionic strength and the quicker solubilization at lower salt concentrations prompted us to look for possible specific types of interaction which stabilize the folded structure of chromatin, despite discontinuities on the DNA. To this end, we further digested in solution folded chromatin solubilized from nuclei after 15 min of digestion with micrococcal nuclease. The sedimentation analysis in 140 mM KCl at different times of digestion (Figure 5A, a–f) shows that the mass distribution of chromatin did not change gradually to smaller s values. Chromatin in the maximum of the original distribution (corresponding to an average of 25 nucleosomes) sedimented with a constant s value, although the advancing boundaries became hypersharper. Concomitantly, nucleosome monomers and small oligomers were produced. The decrease in relative amounts of the fast sedimenting material during the digestion was partly due to precipitation (Table I) as determined by DNA analysis of the pellet and supernatant fractions. We have also observed that when even larger chromatin fragments are digested under these conditions there is an early shift in the s value until the majority of the material reaches a plateau in the s value at around 100 S (not shown). A similar phenomenon was observed with long chromatin fibers extracted at 40 mM NaCl (procedure 1) after raising the salt concentration to 100 mM NaCl (parts B and C of Figure 9). This behavior depends on the presence of H1 and H5 in chromatin. When H1,H5-depleted chromatin was digested at 140 mM NaCl (Figure 6A), the s value of the distribution became smaller. In addition, the rate of cleavage was increased several-fold, in agreement with other reports (Noll & Kornberg, 1977; Lawson & Cole, 1979).

The DNA of the material sedimenting with a constant s value (parts B and C of Figure 5) is not intact but is progressively cleaved until an apparent limit is reached that corresponds to an average fragment length of eight to nine nucleosomes. No mononucleosomal and little dinucleosomal DNA was detected in this material.

Stable folded chromatin fibers containing cleaved DNA can also be extracted from nuclei, as seen in the overexposed photograph of the DNA electrophoresis of the original material (Figure 5B, a). Therefore, we think that this behavior is not the result of an artifact occurring in solution but is a reflection of structural features of the chromatin fiber. It can be inferred from the asymmetry of the advancing and trailing boundaries

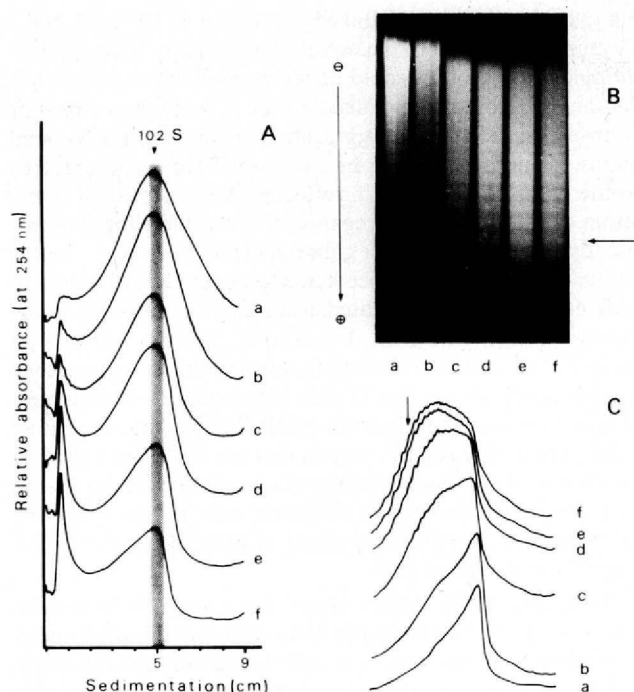


FIGURE 5: Stability of the folded chromatin fiber does not require the covalent continuity of DNA. Chromatin solubilized according to procedure 2 after a 17-min micrococcal nuclease digestion of nuclei was further digested in solution at 140 mM KCl in buffer D with micrococcal nuclease. A fine precipitate which appeared during the digestion was removed by centrifugation (Table I). (A) The supernatants from aliquots taken at different times were analyzed by centrifugation in sucrose gradients for 100 min. Times of digestion with the nuclease were (a–f) 0, 10, 25, 40, 60, and 80 min, respectively. (B) DNA from the chromatin sedimenting with the constant s value in the experiment shown in (A) (shadowed) was analyzed by electrophoresis in 0.7% agarose slab gels. (a–f) correspond to the time of the nuclease digestion: 0, 10, 25, 40, 60, and 80 min, respectively. (C) Densitometric tracings of the DNA electrophoresis shown in (B). Direction of migration is from right to left. The average chain length of the DNA distribution in e and f (B and C) corresponds to a length of eight to nine nucleosomes. The arrow points to the position of pentanucleosomal DNA.

of the DNA-cleaved fiber (Figure 5A) that its stability is at least partly dependent on chromatin concentration. This is supported by the results shown in Figure 6B which compares the sedimentation profiles of nuclease-digested chromatin fibers before (a) and after (b) reaction with glutaraldehyde. Despite the fact that a relatively high proportion of monomeric nucleosomes, and to a lesser extent small oligomers, was irreversibly excised, the cross-linked chromatin fibers displayed a more symmetrical distribution and higher s values (Figure 6B, b). DNA analysis also indicated that the proportion of small oligomers throughout the distribution of cross-linked chromatin was increased, with the exception of the monomers. The changes in the sedimentation profile introduced by the cross-linker contrast with the lack of effect of glutaraldehyde on chromatin which had not been further digested in solution with the nuclease (Figure 4A). These results suggest that an important proportion of the chromatin does not lose its folded structure even after the introduction of discontinuities on the DNA. Whatever the structural implications of the DNA-cleaved folded fiber may be (see Discussion), the stability of such a structure can be used as proof for the superstructure of chromatin (see below).

The liberation of nucleosome monomers and small oligomers (Figures 5A and 6B) during nuclease digestion was further studied. Folded chromatin was digested with micrococcal nuclease and fractionated by sedimentation in sucrose gra-

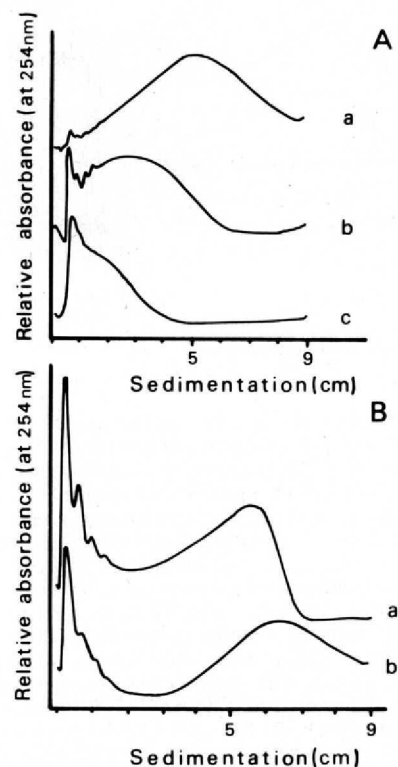


FIGURE 6: Effect of H1 and H5 removal and glutaraldehyde cross-linking. (A) Time course of micrococcal nuclease digestion of H1,H5-depleted chromatin. Histones H1 and H5 were removed as described in the legend to Figure 2B from chromatin which was then dialyzed against 140 mM NaCl in buffer D. Depleted chromatin was digested with micrococcal nuclease for (a–c) 0, 1, and 5 min, respectively, and centrifuged in sucrose gradients for 150 min. (B) Effect of glutaraldehyde cross-linking on folded chromatin containing cleaved DNA. Chromatin prepared according to procedure 2 after 7 min of micrococcal nuclease digestion of nuclei was dialyzed against 140 mM KCl in buffer D (1.8 mg/mL) and was further digested with micrococcal nuclease for 60 min. The supernatant after digestion was cross-linked with 0 (a) and 0.4% (b) glutaraldehyde for 3 h at 0 °C. Equal aliquots (150 μ L) were analyzed by centrifugation in sucrose gradients for 130 min.

dients. Figure 7 shows the DNA size and the relative content of H1 and H5 of the different fractions. No H1 or H5 was detected either free at the top of the gradient or associated with monomers and dimers (C, fraction 1). The relative content of these histones increased continuously with the size of the DNA and the fiber (B and C). There was no sign of an unequal accumulation of H1 or H5 which followed the same trend, except for a relative enrichment of H1 in fractions 2 and 3 (data not shown). A similar distribution of H1 and H5 was also observed in folded fibers without further digestion in solution, although in these cases the amount of small oligomers is much reduced. The distribution of H1 and H5 relative to chromatin size does not appear to be due to their preference for longer DNA but rather for larger chromatin structures regardless of the size of the DNA contained therein. When soluble folded chromatin is digested with nuclease to higher extents (Figure 5), more and more H1 and H5 are found in the fraction of constant s value (Table I) although the DNA becomes shorter. This excess of H1 and H5 may very likely be the reason for the precipitation accompanying the digestion (Table I). A decrease in the ionic strength from 140–100 mM KCl (NaCl) to 5 mM KCl (NaCl) produces an apparent redistribution of H1 and H5 among the different fragments (not shown), with the concomitant solubilization of the chromatin precipitated during digestion. Therefore, we suggest that the reason for the excision of small oligomers from

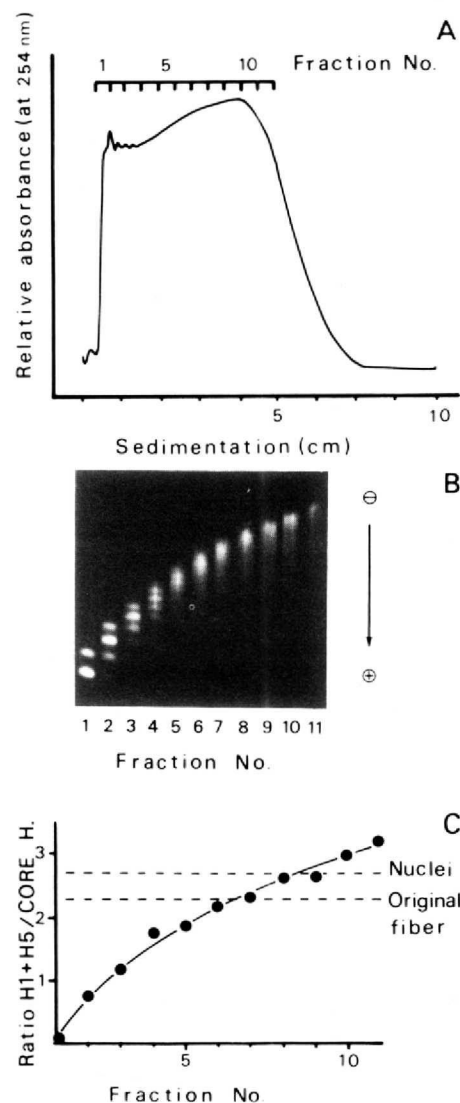


FIGURE 7: Accumulation of H1 and H5 as a function of the size of the folded chromatin. (A) Chromatin extracted from nuclei according to procedure 1 after 30 min of micrococcal nuclease digestion (30 units/mg of DNA) was dialyzed against 100 mM NaCl in buffer D and further digested for 40 min with micrococcal nuclease. The supernatant of the digestion was sedimented in sucrose gradients for 100 min. (B) Electrophoresis in a 0.7% agarose slab gel of DNA from chromatin fractionated as indicated in (A). The fastest moving electrophoretic band in lane 1 corresponds to the DNA of nucleosome monomers. (C) Aliquots of the fractions containing the same amount of chromatin DNA were analyzed by NaDodSO₄ gel electrophoresis. The relative content of histones H1 and H5 of each chromatin fraction, determined by densitometry, is plotted as a function of the distance sedimented. The fractions correspond to those analyzed in (B).

larger structures is their effective lack of H1 and H5.

Factors Influencing the Stability of the Folded Structure of Chromatin. The relaxation experienced by chromatin between 140–100 and 40 mM NaCl results in the destabilization and crumbling of the DNA-cleaved folded structures (Figure 8). In addition to the expected overall decrease in the *s* value occurring at 40 mM NaCl (Figure 2A), it is clear that the cut oligomers can no longer interact to form larger structures as they do at 100 mM NaCl (Figure 8a) and they are released into the solution (Figure 8b).

The folding \rightleftharpoons unfolding transition of relatively long chromatin fragments is reversible insofar as the fibers contain long DNA (Figure 9A, b and c), whereas this reversibility does not occur in the DNA-cleaved folded fiber. This is observed when fibers digested with nuclease at 100 mM NaCl can no

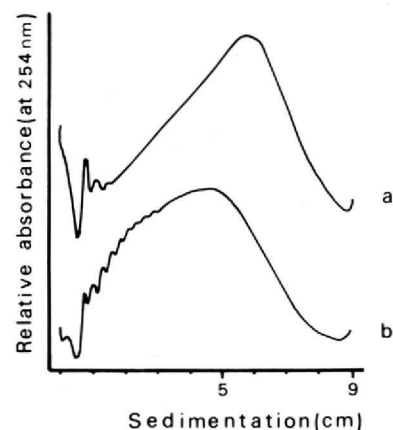


FIGURE 8: Destabilization of the DNA-cleaved chromatin fiber at intermediate ionic strength. Chromatin solubilized from nuclei according to procedure 2 after 17 min of micrococcal nuclease digestion was dialyzed against 100 mM NaCl in buffer D and further digested with micrococcal nuclease for 20 min. Equal aliquots of the supernatant after the digestion were dialyzed against 100 (a) and 40 mM NaCl (b) in buffer D and sedimented in sucrose gradients for 130 min. The *s* value at the peak of the distribution was 105 (a) and 64 S (b).

longer regain the original sedimentation pattern after exposure to 5 or 40 mM NaCl (Figure 9B).

Although we have shown that H1 and H5 appear to have a decisive role in the correct folding and possibly in the stability of the folded chromatin structure, we wanted to determine whether a limited cleavage of these two histones was sufficient to interfere with these processes. Histones H1 and H5 were partly attacked by trypsin at 5 mM NaCl under conditions in which no digestion of the core histones was detected (Figure 9D, a and b). Additional experiments also indicated that the majority, at least, of H1 and H5 tryptic peptides remained bound to the chromatin (unpublished observations). Trypsin-treated chromatin showed at 100 mM NaCl higher compactness (Figure 9C, a and b) than H1,H5-depleted material (Figure 2B, a and b), but its stability, probed with micrococcal nuclease (Figure 9C, c and d), was reduced similarly (Figure 6A) and at the same time the rate of nuclease digestion was also increased several-fold. This behavior contrasts with that of control chromatin (Figure 9C, a and c) which was treated in the same way, except for the omission of the trypsin treatment. These observations suggest a cooperative-like effect of H1 and H5 in the organization of the superstructure of chromatin.

We also investigated the possibility that, once the 23-nm fiber is properly structured, stabilizing contacts involving the core histones are established. This was analyzed by digesting DNA-cleaved folded chromatin with trypsin and correlating the rate of histone cleavage with the crumbling of the structure. It had been reported that trypsin digestion of chromatin leads to the fast cleavage of H1 and to the slower degradation of the charged N termini of the core histones, leaving a limit product thought to be the interacting nonpolar regions of the core histones (Weintraub & Van Lente, 1974). Figure 10A shows that in 140 mM KCl a controlled trypsin treatment of nuclease-digested folded chromatin gradually shifts the original chromatin distribution to that of its smaller components in much the same way as lowering the ionic strength to 40 mM NaCl or KCl. Since the *s* value of the oligomers is not detectably changed, trypsin digestion is mostly affecting the factors which keep together the DNA-cleaved chromatin fiber in its compact configuration. As expected, the DNA fragment distribution of equivalent chromatin fractions (Figure 10B)

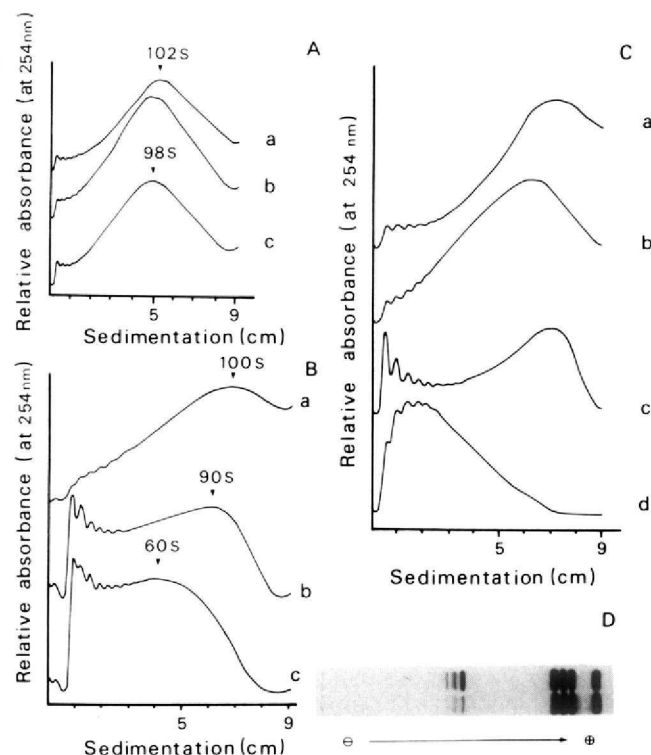


FIGURE 9: Reversibility of the folded \rightleftharpoons unfolded transition of chromatin. (A) Chromatin prepared according to procedure 2 after 17 min of micrococcal nuclease digestion of nuclei was dialyzed against either 100 or 5 mM NaCl in buffer D. The ionic strength of chromatin at 5 mM NaCl was continuously increased up to 100 mM NaCl at a rate of 6.8 mM/h by dialysis. Samples were analyzed by sedimentation in sucrose gradients for 100 min. (a) Original chromatin at 120 mM KCl; (b) same as (a) after dialysis against 100 mM NaCl; (c) refolded chromatin (120 mM KCl \rightarrow 5 mM NaCl \rightarrow 100 mM NaCl). (B) Irreversibility of the salt-dependent transitions of the DNA-cleaved chromatin fiber. Chromatin extracted from nuclei according to procedure 1 after 10 min of micrococcal nuclease digestion was dialyzed against 100 mM NaCl in buffer D (a) and further digested with micrococcal nuclease for 20 min (b). An aliquot of the digested chromatin was dialyzed against 5 mM NaCl and further against 100 mM NaCl in buffer D (c). Samples were sedimented in sucrose gradients for 150 min. (C) Intact histones H1 and H5 are necessary for the folding of the chromatin fibers. Chromatin prepared as in (A) was dialyzed against 5 mM NaCl in buffer D from which PMSF was omitted. An aliquot was digested with trypsin for 10 min at 4 $^{\circ}$ C, and the reaction was stopped with soybean trypsin inhibitor. Both treated and nontreated chromatin samples were dialyzed against 100 mM NaCl in buffer D. Aliquots of the fibers were then further digested with micrococcal nuclease for 3.5 min and sedimented in sucrose gradients for 140 min. (a) Control chromatin; (b) trypsin-digested chromatin; (c) same as (a) after nuclease digestion; (d) same as (b) after nuclease digestion. The *s* value at the peak of the distribution of chromatin was 110 (a) and 80 S (b). (D) Electrophoretic pattern of histones from untreated (a) and trypsin-digested (b) chromatin used in the experiments shown in (C).

shows a loss of the smaller fragments, with a bias toward larger DNA, as digestion proceeded, an opposite trend to that seen during nuclease digestion of the structured 23-nm fiber (Figure 5). Analysis of the histones revealed that they were not cleaved at the same rates. H2A and H2B were either not attacked or attacked only to a very small extent at the last point of digestion, whereas H3 and H4 were cleaved almost in a parallel fashion (Figure 10D); H1 and H5 were cleaved at the fastest rate of all (Figure 10C). These results, although not conclusive with regard to H3 and H4, do rule out that interactions involving only H2A and H2B play a major role in the stability of the folded fiber.

Finally we would like to point out that no other major protein bands, besides those corresponding to the histones,

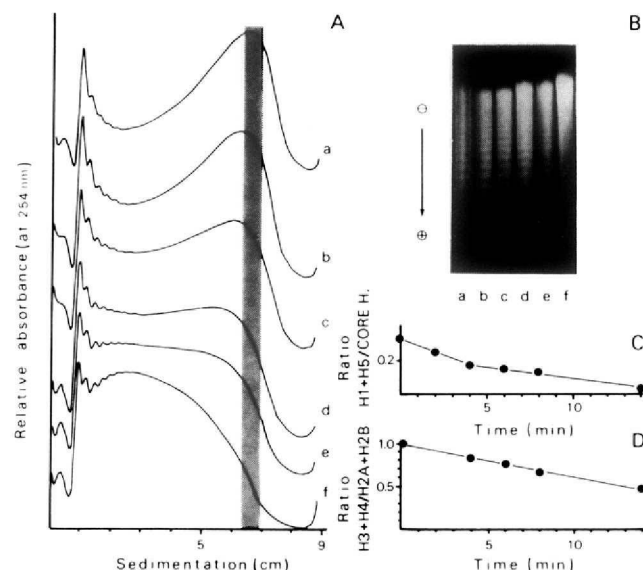


FIGURE 10: Trypsin treatment of chromatin fibers containing cleaved DNA. Chromatin extracted according to procedure 2 after 17 min of micrococcal nuclease digestion of nuclei was dialyzed against 140 mM KCl in buffer D, from which PMSF had been omitted, and further digested in solution with micrococcal nuclease for 60 min. The supernatant from the digestion was treated with trypsin for 0 (a), 2 (b), 4 (c), 6 (d), 8 (e), and 14 (f) min, respectively. Trypsin was inhibited with soybean trypsin inhibitor. (A) Sedimentation profiles of chromatin in sucrose gradients centrifuged for 130 min. The *s* value at the peak of the distribution of chromatin in (a) was 100 S. (B) 0.7% agarose slab gel electrophoresis of DNA from equivalent fractions of the gradients shown in (A) (shadowed). (C and D) Relative rate of cleavage of histones in chromatin by trypsin. Histones from chromatin in equivalent fractions of the gradients shown in (A) (shadowed) were analyzed by electrophoresis. The relative amount of intact histones remaining after the trypsin treatment, determined by densitometry, is plotted as a function of the time of trypsin digestion. Under the conditions of the experiment, histones H2A and H2B were virtually not attacked or only to a very low extent and they were used as reference.

could be seen by electrophoretic analysis of whole chromatin fibers (Figure 9D, a). Only upon overloading were very small amounts of a protein, which may correspond to the HMG group (Goodwin & Johns, 1972), and trace amounts of other proteins detected. In fact, the amounts of HMG and other nonhistone proteins are very low in chicken erythrocytes (Johns et al., 1977; Ruiz-Carrillo et al., 1974). Although we do not a priori exclude a structural role for these proteins, for the reasons given above we consider it unlikely that they are responsible for the gross structural changes described in this work.

Discussion

We have reinvestigated the folded \rightleftharpoons unfolded transition of the structure of chromatin occurring between 0 and 140 mM NaCl with nuclear material solubilized at 140 mM NaCl (or KCl) and produced evidence for its reversibility as determined by hydrodynamic and biochemical methods. This transition requires relatively long DNA and, at least, the full complement of intact histones H1 and H5. From 140 mM to at least 40 mM NaCl the unfolding of chromatin can be visualized as a rather continuous loosening of the compact structure. The existence of a family of superhelical structures of constant diameter and varying degrees of compactness, depending on the concentration of salt, has already been proposed to fit light scattering data of chromatin, although in a range of lower ionic strengths (Campbell et al., 1978).

We think that our results are also compatible with a superhelical organization of the polynucleosomal chain in the higher ordered structure of chromatin as proposed by Finch & Klug (1976).

Divalent cations and polyamines are known to promote conformational changes in nucleic acids and nucleic acid-protein complexes [see, for instance, Gosule & Schellman (1978) and Finch & Klug (1976)]. In our experiments nuclei were isolated and digested in the presence of either or both components which were, however, not included in the solubilized chromatin preparations; an exception was made for Ca^{2+} during the subsequent nuclease digestions. Although we cannot rule out subtle structural effects in chromatin resulting after chelation of divalent cations, there are reports in the literature that show that monovalent cations can substitute the effects of Mg^{2+} , albeit at higher concentrations (Leake et al., 1972; Bradbury et al., 1975). In addition, the similarity in the DNA patterns of chromatin digested *in situ* (in the presence of Mg^{2+}) and in solution (in its absence) (Figure 5B) suggests that the structural features recognized by the nuclease cannot be very different.

The folding of the polynucleosome chain in the superstructure is clearly dependent on the presence of H1-like histones as already pointed out by other workers (Finch & Klug, 1976; Lewis et al., 1976; Renz et al., 1977; Hozier et al., 1977; Thoma & Koller, 1977). Although H1,H5-depleted chromatin also experiences a change in the *s* value with an increase in the ionic strength, the resulting structure is less compact and very unstable, suggesting that in the absence of H1 (H5) the structure has important levels of disorganization. In fact, we have shown that proteolytic degradation of these histones severely impairs the organization and stability of the 23-nm structure. Two major steps, which may arise from two different phenomena, can be distinguished in the unfolded \rightarrow folded transition of chromatin, namely, the increase in the flexibility of the DNA by screening of the phosphate groups by salt and the establishment of histone-histone and/or histone-DNA contacts. The first step must be an important factor in the conformational transition observed between 5 and 40 mM NaCl because the *s* value of naked DNA is strongly influenced in this ionic strength range (Triebel & Reinert, 1971). On the other hand, an increase from 40 to 140 mM NaCl has virtually no effect on DNA (Triebel & Reinert, 1971), whereas chromatin experiences further levels of condensation. The fact that the stability of folded chromatin is abolished at 40 mM NaCl (Figure 8) suggests that specific interactions involving H1 (H5) are needed for the ordered coiling of the polynucleosomal chain, but the stabilizing effects of this organization are not manifested until higher ionic strengths (100–140 mM NaCl). This set of interactions is likely to be responsible for the striking stability of the DNA-cleaved folded fiber, which we have used as a proof for the higher ordered structure of chromatin.

Although the redistribution of H1 and H5 during nuclease digestion of folded chromatin and the accumulation of these histones in the larger structures are not fully understood, it is possible that this observed binding preference may reflect at least some of the factors stabilizing the compact structure. To some extent, H1 and H5 depletion occurs also during digestion of nuclei since we have observed that the shorter the average length of the extracted fiber, the lower its overall content of H1 and H5 (Figure 7C, Table I). The loss of H1 and H5 from the oligomers excised from chromatin digested in solution (Figures 5 and 7) cannot be explained by end trimming of the DNA because at 4 °C the exonucleolytic

activity of the nuclease is much reduced (Noll & Kornberg, 1977; see also Figure 1A). In addition, the exonucleolytic action would not explain the low amount of H1 and H5 in larger oligomers (Figure 7C, fractions 2–7). The small oligonucleosomes released by the nuclease action in solution may originate from disorganized stretches or from the ends of the fibers, which may have lost at least some of their H1 (H5) to the residual nuclear material. This would endow them with a higher susceptibility to nuclease and result in an apparent enrichment of these histones in the residual structure. However, as the digestion proceeds, this effect should be less prominent, especially for the longer material. It is, then, clear that histone H1 (H5) shows a binding preference for the folded material, regardless of the state of the DNA. Therefore, it is conceivable that due to nuclease action some stretches of chromatin which are destabilized and dissociated from the rest of the structure lose their H1 (H5) to the remaining fiber.

We have shown that it is possible to obtain stable fragments of folded chromatin that contain cleaved DNA. Therefore, there must be protein-protein and/or protein-DNA interactions that hold the fiber together. Although histone H1 (H5) is likely to play a role in these interactions, stabilizing contacts involving the core histones, mainly H3 and H4, cannot be ruled out.

The reasons for the apparent limit of the nuclease digestion in the folded fiber (weight average of eight to nine nucleosomes) are not evident. The DNA size in these fibers is close to the proposed nucleosome content of a superbead (Hozier et al., 1977). The biochemical evidence for such a structure was taken from the analysis at 40 mM NaCl of chromatin fragments produced during a time course digestion of chromatin by micrococcal nuclease. From our studies, it is clear that at this salt concentration folded chromatin undergoes a loosening of the structure (Figures 2A and 3), resulting in the instability of the DNA-cleaved fibers (Figure 8). Since the enzyme appears to attack chromatin inside the nucleus in a similar fashion as it does in solution (data not shown), it is not inconceivable that such structures, when extracted at 40 mM NaCl, give rise to an oligomer fragment distribution with a maximum centered around eight to nine nucleosomes and an apparent plateau at 40 S. This could also explain why Hozier et al. (1977) did not find evidence for the presence of oligomeric superbeads.

We propose that the pattern of nuclease digestion of folded chromatin reflects structural features, the limit digestion observed having a relationship to the pitch of a superhelical structure. It is possible that a minimum of one turn is necessary to maintain the ordered fiber, and, therefore, only a specific number of cuts can occur before the structure either falls apart or precipitates.

After submission of this manuscript, Thoma et al. (1979) have reported a systematic electron microscopy study of the salt-dependent structural transitions of chromatin isolated after brief micrococcal nuclease digestion of nuclei. Although in most of their experiments chromatin was exposed to 0.2 mM EDTA during extraction, from their qualitative study they were able to conclude that polynucleosomal chains organize into more or less regular compact fibers of 25-nm diameter at the higher ionic strengths studied. Their results also suggest that if chromatin is organized in superhelical structures, then the pitch remains fairly constant while the number of nucleosomes per turn increases during the process of condensation. They have also shown that H1 is necessary for a more regular and compact organization of the fiber. These results are in agreement with our observations.

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