

INVOLVEMENT OF HISTONE H1 IN THE ORGANIZATION OF THE NUCLEOSOME AND OF THE SALT-DEPENDENT SUPERSTRUCTURES OF CHROMATIN

F. THOMA, TH. KOLLER, and A. KLUG

From the Institut für Zellbiologie, Eidgenössische Technische Hochschule, Hönggerberg, CH-8093 Zürich, Switzerland, and the Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, England

ABSTRACT

We describe the results of a systematic study, using electron microscopy, of the effects of ionic strength on the morphology of chromatin and of H1-depleted chromatin. With increasing ionic strength, chromatin folds up progressively from a filament of nucleosomes at ~1 mM monovalent salt through some intermediate higher-order helical structures (Thoma, F., and T. Koller, 1977, *Cell* 12:101-107) with a fairly constant pitch but increasing numbers of nucleosomes per turn, until finally at 60 mM (or else in ~0.3 mM Mg^{++}) a thick fiber of 250 Å diameter is formed, corresponding to a structurally well-organized but not perfectly regular superhelix or solenoid of pitch ~110 Å as described by Finch and Klug (1976, *Proc. Natl. Acad. Sci. U. S. A.* 73:1897-1901). The numbers of nucleosomes per turn of the helical structures agree well with those which can be calculated from the light-scattering data of Campbell et al. (1978, *Nucleic Acids Res.* 5:1571-1580).

H1-depleted chromatin also condenses with increasing ionic strength but not so densely as chromatin and not into a definite structure with a well-defined fiber direction. At very low ionic strengths, nucleosomes are present in chromatin but not in H1-depleted chromatin which has the form of an unravelled filament. At somewhat higher ionic strengths (>5 mM triethanolamine chloride), nucleosomes are visible in both types of specimen but the fine details are different. In chromatin containing H1, the DNA enters and leaves the nucleosome on the same side but in chromatin depleted of H1 the entrance and exit points are much more random and more or less on opposite sides of the nucleosome.

We conclude that H1 stabilizes the nucleosome and is located in the region of the exit and entry points of the DNA. This result is correlated with biochemical and x-ray crystallographic results on the internal structure of the nucleosome core to give a picture of a nucleosome in which H1 is bound to the unique region on a complete two-turn, 166 base pair particle (Fig. 15). In the formation of higher-order structures, these regions on neighboring nucleosomes come closer together so that an H1 polymer may be formed in the center of the superhelical structures.

KEY WORDS chromatin · nucleosome · histone H1 · higher-order structures · cross-linking

To understand the mechanisms involved in gene expression in higher organisms, a detailed knowledge of the structural organization of chromatin in eukaryotic nuclei is needed. Since chromatin in the large is, by its very nature, only imperfectly ordered, x-ray and neutron diffraction observations permit only limited and indirect interpretation, and the obvious method is that of electron microscopy. There is indeed now a large literature on the electron microscopy of chromatin, so much so that it is becoming unmanageable. Many of the observations are fragmentary and tend to be carried out under one particular set of conditions, so that it becomes almost impossible to compare results obtained with different methods of chromatin extraction, with varying buffers at different ionic strengths, and with different types of specimen preparation for the electron microscope.

Under a wide variety of conditions, nucleosomes themselves can be observed (11, 24, 33) joined together in a chain of nucleosomes (31). The chain of nucleosomes can be compacted in solution to form thick 200- to 300-Å fibers in the presence of small amounts of Mg^{++} (12) or at higher concentrations of monovalent cations (38). These fibers are similar to those found in whole-mount preparations of chromosomes (39) and in sectioned nuclei (7, 4). By electron microscopy, an intermediate higher-order structure has been found at low ionic strength where the nucleosomes are still visible but joined together to form a looser but definite 250-Å fiber (53). What we need to know is how the nucleosomes are joined together and what the arrangement of nucleosomes is in these higher-order structures.

The formation of both this first intermediate and higher-order structures is dependent on the presence of histone H1. It had been known for some time that histone H1 is somehow implicated in the condensation of chromatin (e.g. references 26, 3, and 1), and it was subsequently shown that H1 is necessary for the formation of the 300-Å-thick fibers of soluble chromatin (12, 38) and for their preservation in nuclei (4). This supports the specific suggestion made by Finch and Klug (12) that H1 maintains the higher-order coiling of a filament of nucleosomes into a helix or solenoid of pitch 110 Å. If this is indeed what H1 does, the next questions are: where is H1 located and how

does it contribute to the organization of the higher-order structures?

In this work, which extends the studies cited above from our two laboratories, we have set out to provide a reliable basis for answering these questions by carrying out a set of systematic electron microscope observations. Since H1 is a molecule too small to be observed directly in the electron microscope using routine preparation procedures, we have chosen to make a comparison of chromatin containing H1 and H1-depleted chromatin under strictly parallel conditions. For these studies, it is necessary to use chromatin fragments prepared by nuclease digestion, a method which retains the native state of the chromatin as judged by the periodic gel pattern given by further nuclease digestion (29). Such chromatin has been shown to undergo significant structural changes as followed by both x-ray diffraction and electron microscopy (12, 48, 53), whereas chromatin prepared by the older methods which involve a shearing step does not behave in this definite manner (cf. also reference 8). Since higher-order structures of the kind to be expected in chromatin are easily lost or damaged, it has also been necessary to pay particular attention to the fixation and spreading conditions when preparing the specimens for electron microscopy, and to eliminate artifacts, so that one can be reasonably sure that the forms observed on the grid reflect the structures present in solution.

We show that, upon increasing the ionic strength, chromatin containing H1 in solution folds progressively from a loose nucleosome filament until, at ~60 mM NaCl, it forms a fiber of ~250 Å diameter, after which no further significant changes take place. The thick fiber has the form of a flexible but definite superhelix (or solenoid). By correlating the electron microscope observations with data on the light scattering from chromatin as a function of ionic strength (5), one can estimate the number of nucleosomes per turn in the progressively more condensed chromatin. The data support the occurrence of the intermediate higher-order structure observed by Thoma and Koller (53) and culminate in a value of about six nucleosomes per turn for the 250-Å fibers, a value which agrees with that deduced by Finch and Klug (12) for their superhelical (or solenoidal) model with a pitch ~110 Å.

The parallel experiment with H1-depleted chromatin shows a less-pronounced condensation with increasing ionic strength, and no regular definite

fibers are formed. Nevertheless, the observations have proved very useful. In contrast to chromatin containing H1, at very low ionic strength the nucleosomes of H1-depleted chromatin unravel into extended linearized filaments, indicating that H1-depleted nucleosomes are less stable than nucleosomes containing H1. At slightly higher, but still low, ionic strengths, individual nucleosomes are visible and a comparison of the fine details of this chromatin without histone H1 and that with H1 enables one to deduce where the H1 (or part of it) is located on the nucleosome. This conclusion agrees with that drawn from the results of x-ray crystallographic and biochemical studies. By following the series of condensed forms induced by increasing ionic strength, one can also deduce where the H1 might lie in the 250-Å-diameter fiber and how it is involved in organizing it.

MATERIALS AND METHODS

Materials

Benzyltrimethylammonium chloride (BAC) (*n*-alkyl mixture: C₁₂H₂₅ 60% and C₁₄H₂₉ 40%) was a gift from Bayer (Leverkusen, Germany). Micrococcal nuclease was purchased from Worthington Biochemical Corp. (Freehold, N. J.). All other chemicals (analytical grade) were obtained from Merck Chemical Div., Merck & Co., Inc. (Rahway, N. J.).

Methods

PREPARATION OF CHROMATIN: Nuclei were isolated from rat liver as described by Hewish and Burgoyne (18). For the extraction of chromatin, a procedure similar to that of Thomas and Kornberg (55) was followed as described earlier (53). Chromatin was briefly digested by micrococcal nuclease and solubilized by lysis of the nuclei in 0.2 mM EDTA (pH 7.0). In some experiments, the lysis of the nuclei was performed as described elsewhere (38, 51). No differences were found in the behavior of the chromatin prepared in the three different lysis buffers used. The chromatin fragments of a single preparation varied in size (~20–100 nucleosomes). The significant observations described below were made on the larger fragments.

For the removal of histone H1, the chromatin solution was adjusted to 50 mM sodium phosphate, pH 7.0, 0.2 mM EDTA, and 100 mM NaCl and stirred in the presence of one-quarter of the volume of the ion exchange resin AG50W-X2 (Bio-Rad Laboratories, Richmond, Calif.) on ice for 90 min. The resin was pelleted at 500 g for 5 min; the supernate contained H1-depleted chromatin (53, and Fig. 1).

COURSE OF THE EXPERIMENT: All steps in the following experiments were made at pH 7.0. Chromatin and histone H1-depleted chromatin were divided into aliquots which were dialyzed between 10 and 15 h at 4°C in parallel against the following solutions: 0.2 mM EDTA; 0.2 mM EDTA, 1 mM triethanolamine chloride (TEACl); 0.2 mM EDTA, 5 mM TEACl; 0.2 mM EDTA, 5 mM TEACl, plus concentrations of NaCl varying from 0 to 100 mM. In certain experiments, NaCl was replaced by KCl or up to 2 mM MgCl₂. An aliquot of each sample was then withdrawn for protein analysis by SDS gel

electrophoresis. The histone bands (Fig. 1) agree well with those of published gel patterns (cf. reference 29). No significant proteolytic degradation could be observed.

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS: SDS 15% polyacrylamide slab gels were run as described by Laemmli (22), except that the running gel and the electrode buffer concentrations were doubled. Chromatin samples were adjusted to 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol, and traces of bromophenol blue in the stacking gel buffer and heated for 3 min at 95°C before being applied to the gels.

ANALYTICAL ULTRACENTRIFUGATION: Sedimentation velocity experiments were performed in an MSE Scientific Instruments LTD (Crawley, Sussex England) Analytical Ultracentrifuge Mk II, equipped with an Ultraviolet Scanner. Sedimentation was done at 5°C and scans were taken at 260 nm every 10 min. Weight average sedimentation coefficients were calculated by numerical integration through the boundary to the plateau on each scan, to obtain the weight average position of the boundary, and linear regression analysis of the natural logarithm of the radius of the boundary upon time. These values were corrected for solvent density and viscosity to that of water at 20°C as described by Svedberg and Pedersen (52).

The results on a particular sample, both unfixed and fixed in glutaraldehyde at different ionic strengths, are shown in Fig. 2.

ELECTRON MICROSCOPY: For electron microscopy, the samples were fixed at 4°C for at least 15 h by adjusting the solutions in the corresponding buffers to 0.1% glutaraldehyde and used within 2 d. For spreading, the fixed samples were diluted at room temperature with the corresponding fixation buffers to an A₂₆₀ of 0.02–0.06, and BAC (57) was added from a stock solution in water (0.2 g/100 ml) to a concentration of 2 × 10⁻⁴% (~7 × 10⁻⁴ mM). After 30 min, droplets of 5 μl were applied to carbon-coated grids fixed on a sheet of Parafilm (American Can Co., Greenwich, Conn.). Adsorption of the chromatin fibers to the supports was allowed to take place for 5 min. The grids were washed with redistilled water for 10 min, dehydrated for 2–3 s in ethanol, and blotted dry on filter paper. For contrast enhancement, the grids were rotary-shadowed at an angle of 7° using carbon-platinum evaporated from an electron gun. Samples were examined in a Siemens electron microscope 101 at × 20,000. For the magnification calibration, a carbon grating replica grid from Balzers Union (Lichtenstein) was used.

The carbon support films were produced by evaporating carbon from a baked electron gun onto freshly cleaved mica at a vacuum below 5 × 10⁻⁶ torr. The films were floated off on redistilled water and allowed to settle down on 400-mesh copper grids.

In certain control experiments the specimens were freeze-dried. After specimen adsorption the grids were washed either with buffer solutions or with redistilled water. They were then dropped directly into liquid nitrogen previously poured into cylindrical depressions of a cooled copper block. The block was covered with a precooled stainless-steel mesh and transferred onto the cooling table of a Balzers freeze-etching machine (BAE 300). Warming of the block to room temperature took ~12 h at a vacuum of ~10⁻⁶ torr.

RESULTS

Ionic Strength-dependent Condensation of Chromatin Containing H1

Chromatin freshly extracted from the rat liver was divided into aliquots adjusted to different salt

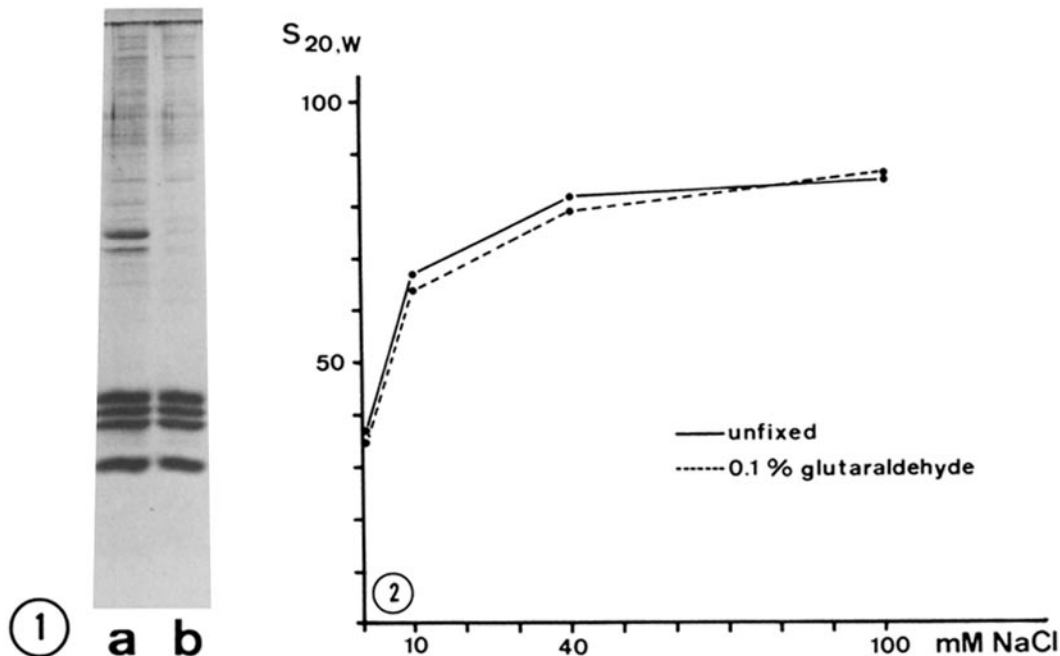


FIGURE 1 SDS polyacrylamide slab gels of (a) chromatin, and (b) H1-depleted chromatin.

FIGURE 2 Sedimentation constants of a particular chromatin preparation, fixed and unfixed, as a function of ionic strength (Courtesy of P. J. G. Butler).

concentrations. Glutaraldehyde was then added to a concentration of 0.1% and fixation was performed at 4°C for at least 15 h before the specimens were prepared for electron microscopy. Fig. 3 illustrates the general appearance of chromatin samples from a single experiment in which samples were fixed in increasing ionic strength of NaCl. The increase in condensation with increasing salt is obvious in this series. When NaCl was replaced by KCl, the results were indistinguishable and are therefore not shown. This experiment has been repeated many times with the same results. There is no clear-cut division between the forms visible at two neighboring ionic strengths but rather a continuous graduation, although the appearance does change at an ionic strength of ~10–20 mM, when individual nucleosomes are no longer clearly separated. Fig. 4 shows micrographs selected from different experiments to bring out particular points of structural detail.

At very low ionic strength (0.2 mM EDTA and 1 mM TEACl or less), chromatin appears as relaxed filaments of nucleosomes with a width of one to two nucleosomes (Figs. 3a and 4a). The nucleosome core is a flat particle of dimensions $\sim 110 \times 110 \times 57 \text{ \AA}$ (13), and our micrographs

suggest that the nucleosomes are preferentially adsorbed to the supporting film on their faces, since the nucleosomes usually appear round in shape (Fig. 4a and b). The nucleosomes are connected by thin filaments which are considered to be part of the internucleosomal linker DNA. Frequently, the DNA seems to enter and to leave the nucleosomes at sites fairly close to each other, giving rise, in places, to an open zigzag appearance of the whole fiber (Fig. 4a). On a specimen fixed in 1 mM TEACl and 0.2 mM EDTA, the nucleosome center-to-center distance, measured over 700 distances, was $192 \pm 42 \text{ \AA}$, corresponding to ~ 55 –60 base pairs (bp). This value gives a rough idea of the maximum possible linker length. The minimum possible linker length would be $\sim 80 \text{ \AA}$ or 25 bp, calculated by subtracting the nucleosome diameter of 110 Å.

When fixation is performed in 5 mM TEACl and 0.2 mM EDTA (Figs. 3b and 4b), the fibers are ~ 200 –250 Å in diameter. Again, they appear relaxed and flat, but the nucleosomes approach more closely than at 1 mM TEACl. Generally, no DNA-like filaments can be resolved connecting the nucleosomes, the latter being arranged in two parallel rows, as though the open zigzag (Fig. 4a)

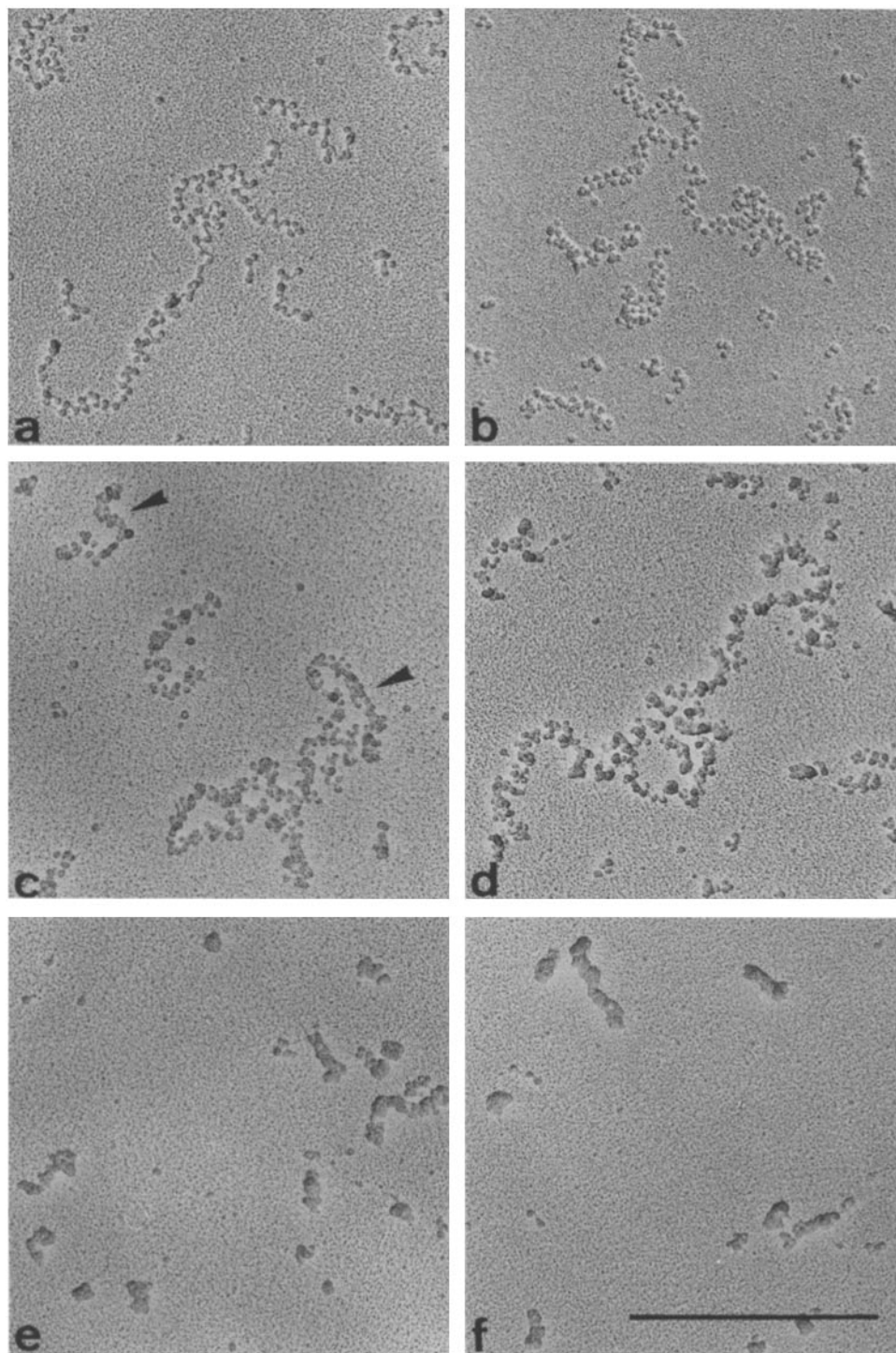


FIGURE 3 Ionic strength dependence of the appearance of chromatin in the electron microscope. Aliquots ($8 A_{260}$ U/ml) from the same chromatin preparation were fixed in 0.1% glutaraldehyde at 4°C for 15 h in the following solutions: (a) 0.2 mM EDTA (pH 7.0); (b) 5 mM TEACl (pH 7.0) and 0.2 mM EDTA; (c) 10 mM NaCl, 5 mM TEACl (pH 7.0), and 0.2 mM EDTA; (d) 20 mM NaCl, 5 mM TEACl (pH 7.0), and 0.2 mM EDTA; (e) 40 mM NaCl, 5 mM TEACl (pH 7.0), and 0.2 mM EDTA; (f) 60 mM NaCl, 5 mM TEACl (pH 7.0), and 0.2 mM EDTA. For spreading, the samples were diluted to an A_{260} of 0.02–0.06 in the fixation buffer containing in addition $2 \times 10^{-4}\%$ BAC. The arrow in c points to a region in which the nucleosomes approach closely to give the impression of a loose helical structure. Bar, 5,000 Å. $\times 70,000$.

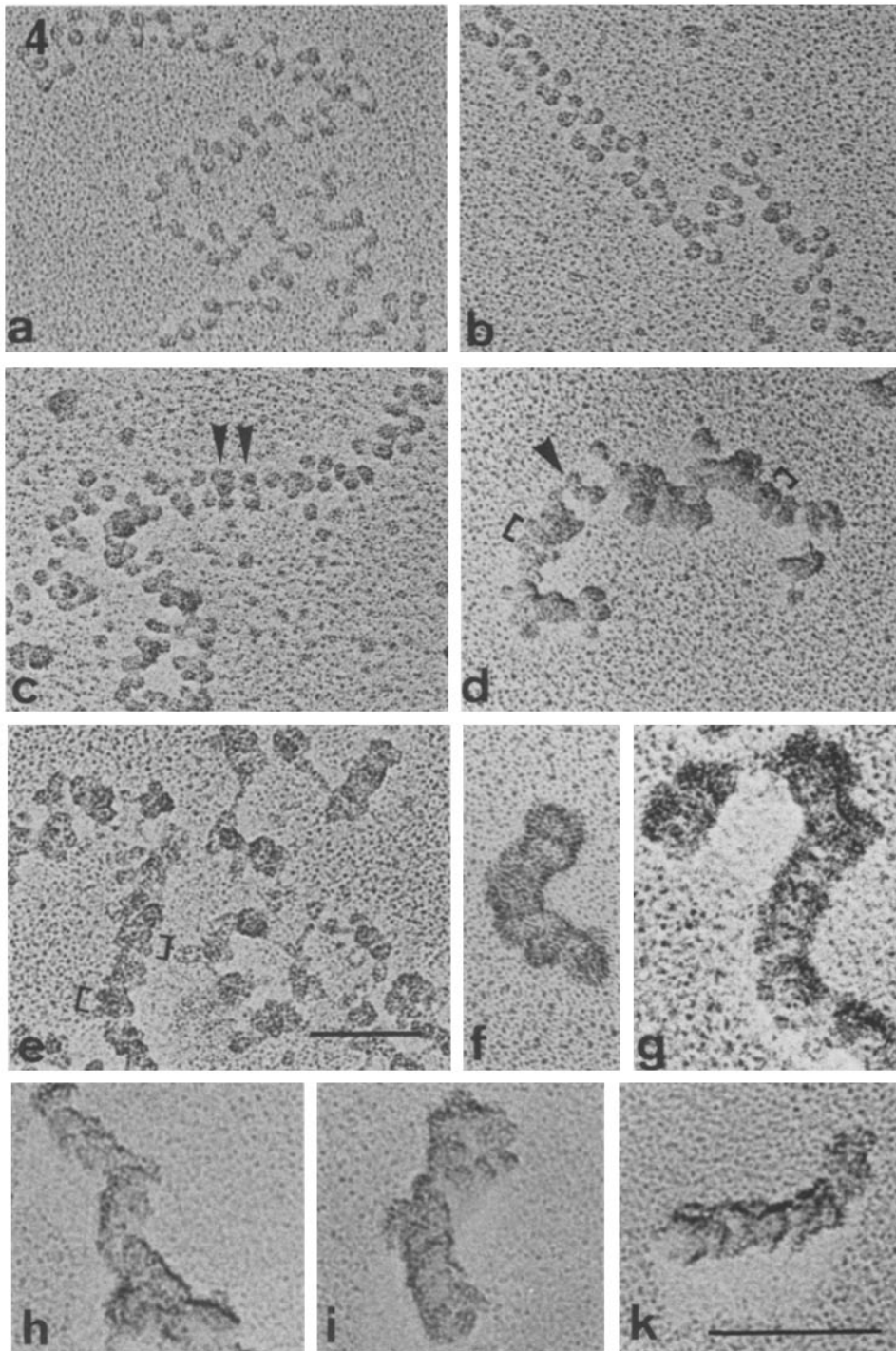


FIGURE 4 Selected high magnification micrographs of chromatin fibers fixed at different ionic strengths. Fixation was performed in: (a) 1 mM TEACl (pH 7.0), 0.2 mM EDTA; (b) 5 mM TEACl (pH 7.0), 0.2 mM EDTA; (c) same as b; (d) 40 mM NaCl, 5 mM TEACl (pH 7.0), 0.2 mM EDTA; (e) same as in d, but freeze-dried after adsorption, then washed on water, dehydrated in ethanol, and air dried; (f-k) 100 mM NaCl, 5 mM TEACl (pH 7.0), 0.2 mM EDTA. a-g Were rotary shadowed with carbon platinum; h-k were shadowed at two fixed angles. Arrows in c and d point to a row of nucleosomes arranged approximately perpendicular to the fiber axis. Brackets in d and e indicate that helical structures tend to fall apart irregularly into individual turns or pair of turns. Bar, 1000 Å. (a-e) $\times 160,000$; (f-k) $\times 240,000$.

had now closed up (Fig. 4*b*) (see also reference 53). Arrows in Fig. 4*c* indicate sites where rows of about three nucleosomes are arranged more or less perpendicular to the fiber axis. Such findings suggest that in solution the nucleosomes may be arranged in helical turns, but on the grid there is the tendency for the nucleosomes to fall on their flat faces, giving rise to the zigzag appearance. Allowing for this bias, one concludes that in solution there is a helix with about two to three nucleosomes per turn. A similar zigzag appearance has been described by Oudet et al. (33) in cellular chromatin examined by lysing nuclei on a grid, under conditions difficult to control.

When 10 mM NaCl is added to the 5 mM TEACl buffer before fixation, the zigzags disappear in favor of a more compact fiber with a diameter of ~ 250 Å (Fig. 3*c*). This is even more evident at 20 mM NaCl (Fig. 3*d*). Again, in certain regions nucleosomes approach closely to give the impression of a loose helical structure that could have three to four nucleosomes per turn (arrows in Fig. 3*c*). The turns are irregular and can be separated by distances up to 200 Å, indicating perhaps stronger cross-linking by the fixative within a turn than between turns. The closest distance of approach between turns is ~ 100 Å or one nucleosomal diameter.

Between 20 (Fig. 3*d*) and 40 mM NaCl (Fig. 3*e* and Fig. 4*d* and *e*), a more striking condensation of the chromatin fiber is observed. At 40 mM, the fibers are irregular and inhomogeneous in shape (Fig. 4*d* and *e*), ~ 250 Å in diameter, and individual nucleosomes are hard to see. Compared to the fibers observed at lower ionic strength, the fibers have a clear three-dimensional appearance with cross-striations ~ 100 – 150 Å apart, which suggest a helical structure composed of closely packed nucleosomes. About three nucleosomes are visible across the fiber, implying about four to six nucleosomes per helical turn (arrow in Fig. 4*d*). Often, globular condensations of, very roughly, 200–250 Å in size (Fig. 3*e* and Fig. 4*d* and *e*) are found along the length of the fiber, similar to the “superbeads” described by Hozier et al. (19). These condensations can often be seen to be composed of two helical turns (brackets in Fig. 4*d* and *e*).

A further condensation of the fibers (Fig. 3*e* and *f*) occurs between 40 and 60 mM NaCl. The fibers appear compact along the lengths; however, irregularities and distortions are evident. Upon increasing the NaCl concentration from 60 up to 100 mM, no further change in morphology is

observed. When allowance is made for the amount of deposited platinum and for other measurements on unshadowed specimens positively stained with uranyl acetate, the diameter of these thick fibers appears to be ~ 250 Å. It is characteristic of these fibers that the nucleosomes are mostly not resolved (Fig. 4*f* and *g*). However, an obvious cross-striation (Fig. 4*h*–*k*) was visible in 22% of all the fibers inspected ($\sim 1,000$), again indicating a helical structure. The underlying helical structure is most clearly seen in micrographs such as Fig. 4*k*, where the fiber is pulled out on the grid. A precise measurement of the pitch and the pitch angle is difficult because the thick fibers are frequently bent. Micrographs such as those shown in Fig. 4*h* and *i* give an approximate pitch distance of the order of 110 to 150 Å. The direction of the cross-striations varies in different fibers, or even along the length of the same fiber, so there is no consistent indication of either a left- or right-handed helix (cf. reference 12). Out of 1,240 fibers measured, $\sim 60\%$ suggested a left-handed helix and 40% a right-handed one.

No further change in morphology is observed on going to still higher ionic strengths (e.g. 100 mM NaCl + 50 mM sodium phosphate), but the solutions become turbid, indicating the onset of precipitation of the chromatin. Under such conditions, adsorption methods for electron microscope specimen preparations are no longer reliable.

Ionic Strength-dependent Condensation of H1-depleted Chromatin

The extraction of histone H1 alters the electron microscopic appearance of chromatin dramatically (12, 38, 53), and also its behavior under changing ionic strength (Figs. 5 and 6). These experiments, together with those carried out in parallel on chromatin, allow one to deduce a structural role for histone H1, as well as a possible localization of H1 on the nucleosome and in the thick fibers. As shown in Fig. 1, H1 extraction by resin treatment also removes some of the nonhistone proteins. Since the removal of H1 is quantitatively the most dominant effect of the resin treatment, we will subsequently mainly refer to H1 although it remains unknown whether H1 alone or in combination with one or more nonhistone proteins is responsible for the observations described below.

First, it must be asked whether the procedure for the removal of H1, and also some of the nonhistone proteins (Fig. 1), damages the structure

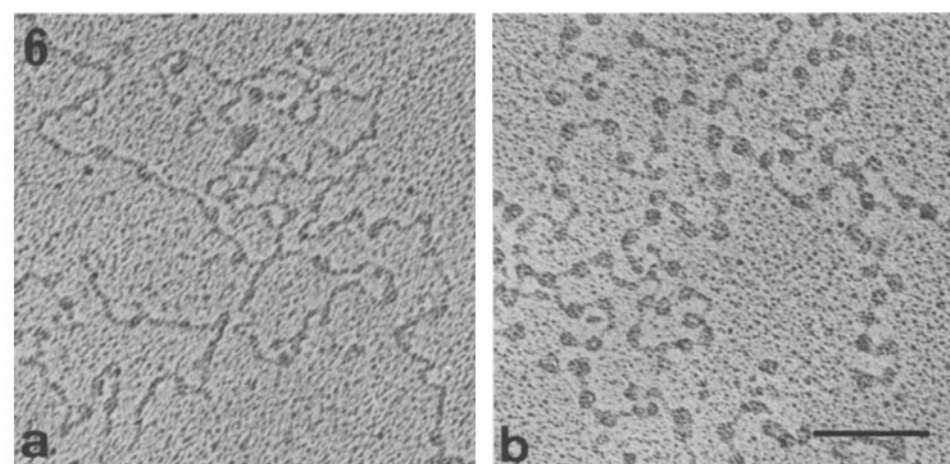
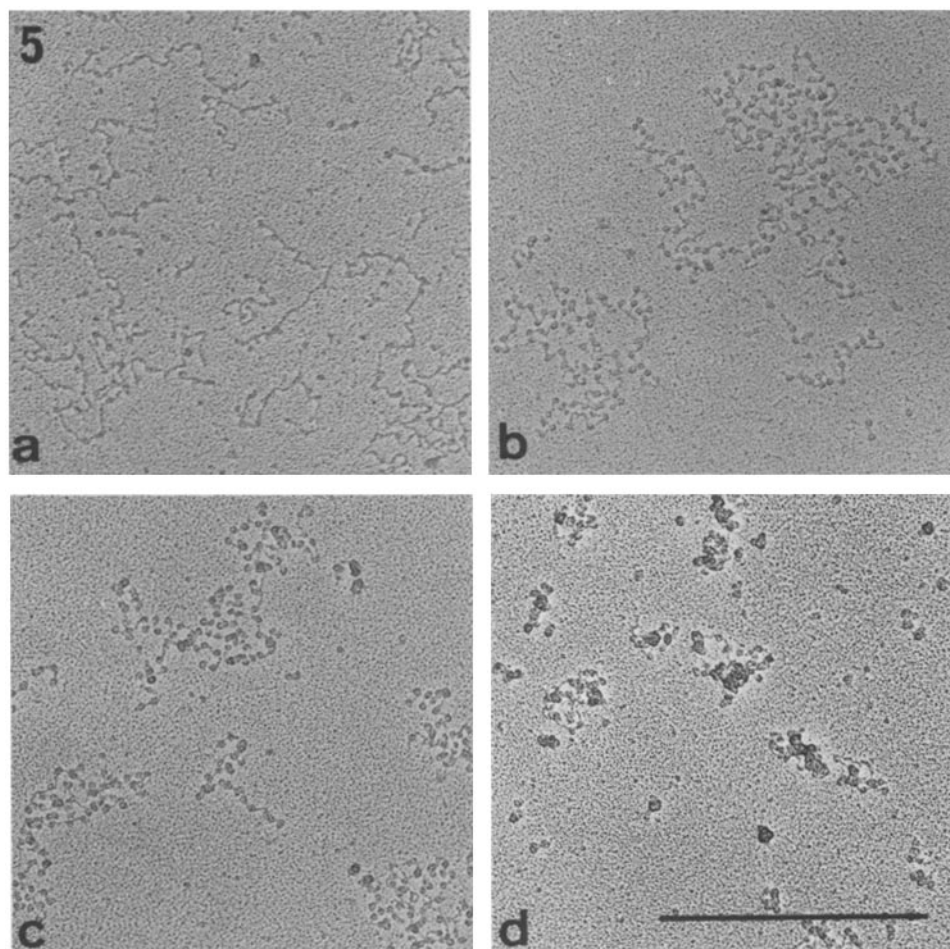


FIGURE 5 The ionic strength dependence of the appearance of H1-depleted chromatin. H1-depleted chromatin samples ($8 A_{260}$ U/ml) were fixed in 0.1% glutaraldehyde at 4°C for 15 h in the following solutions: (a) 1 mM TEACl, 0.2 mM EDTA; (b) 5 mM TEACl, 0.2 mM EDTA, 10 mM NaCl; (c) 5 mM TEACl, 0.2 mM EDTA, 40 mM NaCl; (d) 5 mM TEACl, 0.2 mM EDTA, 100 mM NaCl. For spreading, the samples were diluted to an A_{260} of 0.02–0.06 in the fixation buffer containing in addition $2 \times 10^{-4}\%$ BAC. Bar, 5,000 Å. $\times 70,000$.

FIGURE 6 Selected high magnification micrographs of H1-depleted chromatin treated as in Fig. 5. (a) Same as Fig. 5 a. (b) Same as Fig. 5 b. Bar, 1,000 Å. $\times 150,000$.

left behind or leads to irreversible changes. As a control, histone H1 was removed by column chromatography in the presence of 0.5 M NaCl, 5 mM TEACl (pH 7.0), 0.2 mM EDTA. Subsequent electron microscopy of this material showed no difference from the chromatin depleted in H1 by resin treatment (data not shown). As a further control, salt-extracted H1 together with the accompanying nonhistone proteins were added back to the H1-depleted chromatin by dialysis from salt. The resulting H1-containing chromatin behaved in morphological respects like the untreated chromatin handled in parallel as a control. These experiments form part of a larger study on the reconstitution of chromatin which will be published separately (54, and footnote 1). It would seem, therefore, that the basic nucleosomal repeat is not lost during our procedures for removing of H1, so that the nucleosomal and higher-order structures can be reestablished, unlike the case of reconstitution from extracted histones and DNA which gives only a 140 bp repeat even if H1 is included (50).

Indeed, it has been found² that chromatin stripped of H1 by salt washing at 450 mM NaCl still gives, during the very early stages of micrococcal nuclease digestion, the same 200 bp repeat as the original chromatin. Taken together with the electron microscope experiment, this means that the remaining histones have not redistributed themselves during the H1 removal, unlike the situation when higher salt concentrations are used for the stripping (53).

H1-depleted chromatin fixed at very low ionic strength (in 1 mM TEACl, 0.2 mM EDTA and below) has mainly lost the nucleosomal appearance (Figs. 5*a* and 6*a*), and extended filaments are seen. The irregular thickness of these filaments indicates that the nucleosomes have been opened up but that the proteins are still covering the DNA. Since a parallel sample of H1-containing chromatin under the same ionic strength conditions shows nucleosomes, we conclude that H1, perhaps plus accompanying nonhistone proteins, stabilizes the nucleosomal structure at very low ionic strength. Thus, nucleosomes stripped of H1 undergo structural changes more easily than those which contain histone H1 (cf. Fig. 7).

When H1-depleted chromatin is fixed in pres-

ence of 5 mM TEACl, 0.2 mM EDTA, and 0–10 mM NaCl, chains of nucleosomes appear (Figs. 5*b* and 6*b*), which are clearly different from the definite zigzag-shaped H1-containing chromatin fibers (compare Figs. 5*b* and 6*b* with Figs. 3*a*, 3*b*, 4*a*, 4*b*, 10*a*, 12*a*, and 13). In these chains, the nucleosomes are well separated by DNA-like filaments of uniform thickness (Fig. 6*b*; 53).

Thus, we conclude that, in H1-depleted chromatin, nucleosomes are present in solution at 5 mM salt but not at 1 mM (Fig. 7). Presumably, the reduction in charge repulsion at the higher ionic strength on the chromatin stabilizes the nucleosomal particle. However, this particle is different in the finer details of its appearance from the nucleosomes present in chromatin containing H1, which are best observed in very low ionic strength. In H1-containing chromatin, the DNA enters and leaves the nucleosomes at sites fairly close to each other (Figs. 3*a* and 4*a*), whereas in the nucleosomes of H1-depleted chromatin the DNA tends to enter and leave on more or less opposite sides (Fig. 6*b*). The H1-containing nucleosome is responsible for the zigzag appearance of the corresponding fiber. After removal of H1, the “beads-on-a-string” type of filament appears and these show zigzags only very rarely. Such occasional zigzag occur clearly at random and are not a structural feature as they are in the H1-containing fiber, and are most likely caused by the chance way in which the filament was adsorbed to the supporting grid. (Compare the appearance of the filaments in Fig. 6*b* with that in Fig. 4*b*; see also Fig. 9 and reference 53).

Therefore, we conclude that H1 must be located on the nucleosome at a place where it can bind to the incoming and outgoing DNA. The various forms described for chromatin with and without H1 at low ionic strengths are summarized as a montage in Fig. 7.

When H1-depleted chromatin is fixed at higher ionic strengths, the beads-on-a-string type of filament with a diameter of one nucleosome is still present up to ~40 mM NaCl (Fig. 5*c*), although the filament itself does not appear to meander so far over the grid. The average nucleosome center-to-center distance of H1-depleted chromatin fixed at 10 mM NaCl was $240 \pm 110 \text{ \AA}$ (509 measurements) or $\sim 70 \pm 35 \text{ bp}$.

When fixation is performed above 40 mM NaCl (or KCl), the H1-depleted chromatin condenses much more, but into irregular “clumps” (Fig. 5*d*), and no well-defined fibers of a preferred diameter

¹ Thoma, F., and Th. Koller. Manuscript in preparation.

² Lutter, L. C., and D. Rhodes. Unpublished results.

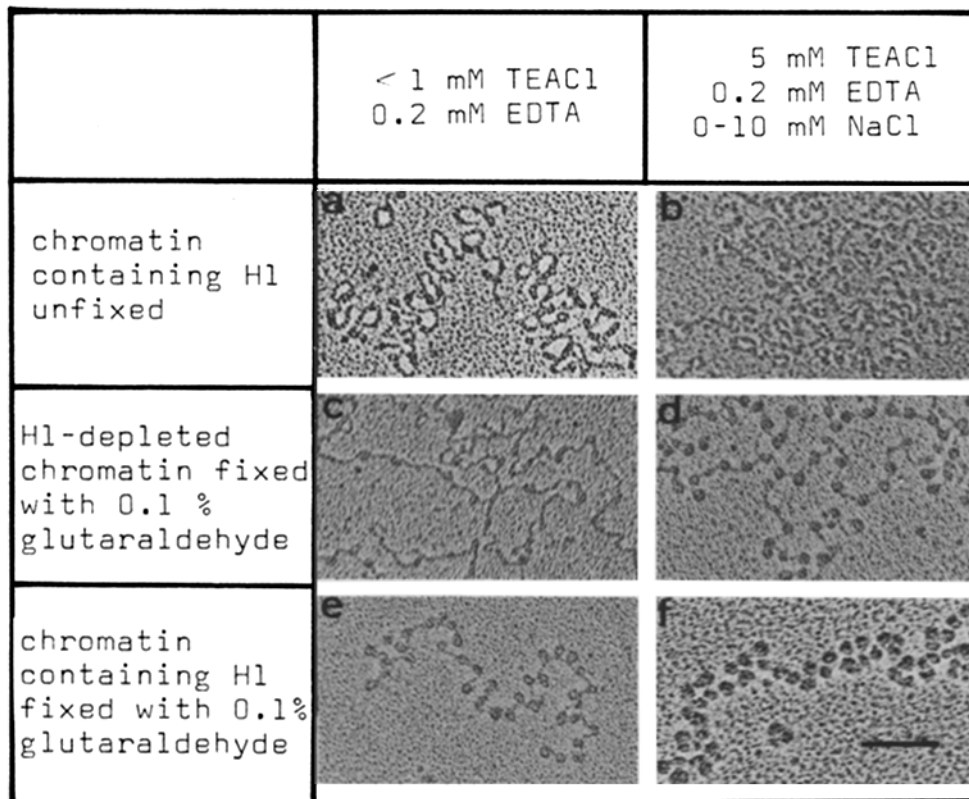


FIGURE 7 Montage summarizing the fibers found at low and very low ionic strengths. Bar, 1,000 Å. $\times 100,000$.

are seen, such as those formed by chromatin containing H1 (Fig. 3*f*). In certain less-folded regions of these irregular clumped forms, beads-on-a-string can still be seen. This type of condensation should be viewed in the light of the findings of Vollenweider et al. (58) who showed that purified DNA spontaneously takes up a superstructure at sodium acetate concentrations of 100 mM and above. Presumably, the cations condense H1-depleted chromatin by inducing DNA superstructure, probably by binding to phosphate groups that are not neutralized by histones. However, the condensation is not so pronounced or, more important, so definite as when H1 is present, so we conclude that histone H1 is necessary for the organization of definite higher-order structures.

Mixing Experiments

The question arises, whether the different forms of chromatin observed on the grid reflect the morphology of chromatin and H1-depleted chromatin in solution or whether they only represent

changes in the conditions of fixation and adsorption.

Fixation works both at very low ionic strength and at low ionic strength, since in unfixed chromatin (with and without H1) the nucleosomes unravelled into extended filaments (Fig. 7*a* and *b*), but after fixation nucleosomes are visible in chromatin containing H1 (Fig. 7*e* and *f*) and in H1-depleted chromatin (>5 mM TEACl) (Fig. 7*d*).

The most striking demonstration that the different types of fibers seen in Figs. 3–6 are not adsorption or preparation artifacts comes from experiments in which chromatin samples fixed under different ionic strength conditions were mixed in the same spreading solution and so prepared on the same grid. Such an experiment is shown in Fig. 8 where chromatin fixed in 1 mM TEACl was mixed with chromatin fixed in 100 mM NaCl, the other extreme of salt concentration. The loosely wound fiber, or nucleosomal filament showing nucleosomes separated by DNA-like thin fila-

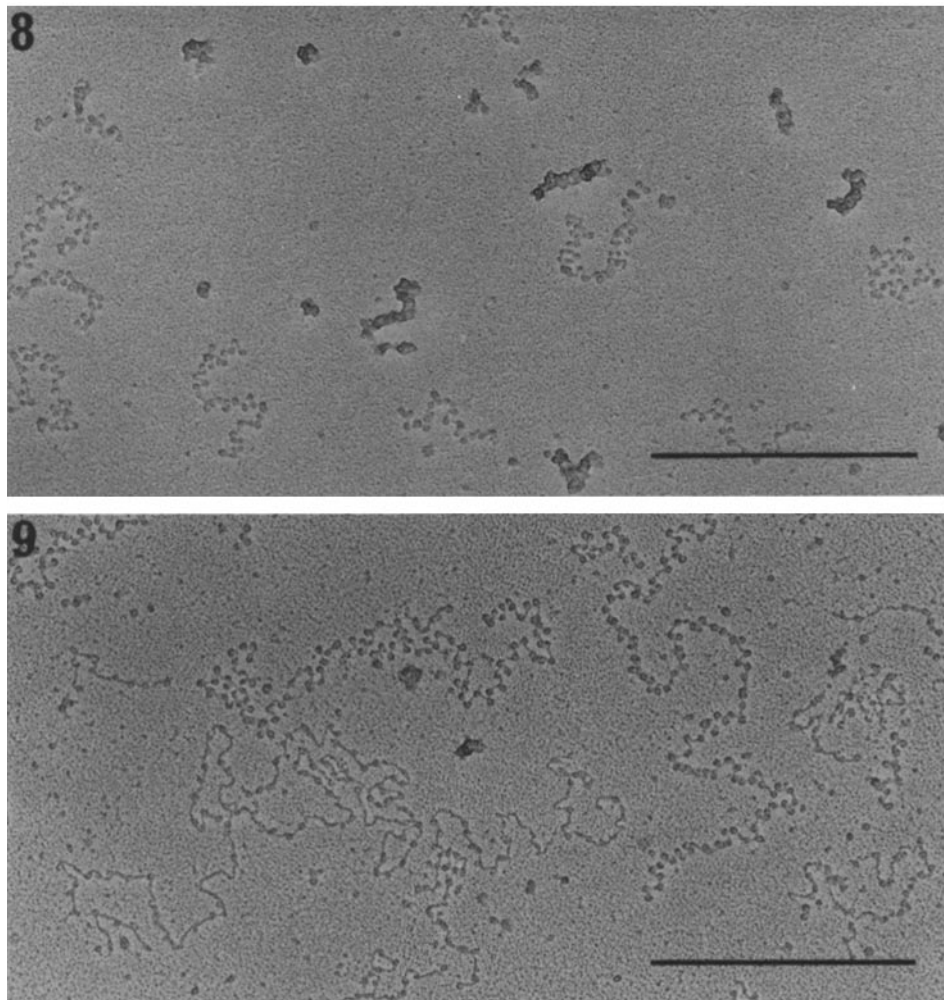


FIGURE 8 Copreparation of chromatin fixed at two different NaCl concentrations. Chromatin ($0.03 A_{260}$ U/ml) fixed in 1 mM TEACl (pH 7.0), 0.2 mM EDTA, and chromatin ($0.03 A_{260}$ U/ml) fixed in 100 mM NaCl, 5 mM TEACl (pH 7.0), 0.2 mM EDTA were mixed in the same spreading solution containing 1 mM TEACl, 0.2 mM EDTA, 0.1% glutaraldehyde, and $2 \times 10^{-4}\%$ BAC. Bar, 5,000 Å. $\times 70,000$.

FIGURE 9 Copreparation of chromatin and H1-depleted chromatin. Chromatin ($0.03 A_{260}$ U/ml) fixed in 1 mM TEACl, 0.2 mM EDTA, and H1-depleted chromatin ($0.03 A_{260}$ U/ml) fixed in the same buffer were mixed in the same spreading solution which contained 1 mM TEACl, 0.2 mM EDTA, 0.1% glutaraldehyde, and $2 \times 10^{-4}\%$ BAC. Bar, 5,000 Å. $\times 70,000$.

ments, characteristic of chromatin fixed at very low ionic strength, and thick fibers, characteristic of chromatin fixed at high ionic strength, are recognized on the same micrograph.

The same type of experiment has also been done with all the chromatin samples studied. Fig. 9 shows, for example, a copreparation of chromatin fixed in 1 mM TEACl, 0.2 mM EDTA and of H1-depleted chromatin fixed in the same buffer. The

typical chromatin forms described above are easily recognized on the same grid. These experiments indicate clearly that the fibers on the micrographs are representative of chromatin in solution and allow us to compare the different structures in detail.

Effect of Magnesium

Fig. 10 summarizes the effects of magnesium

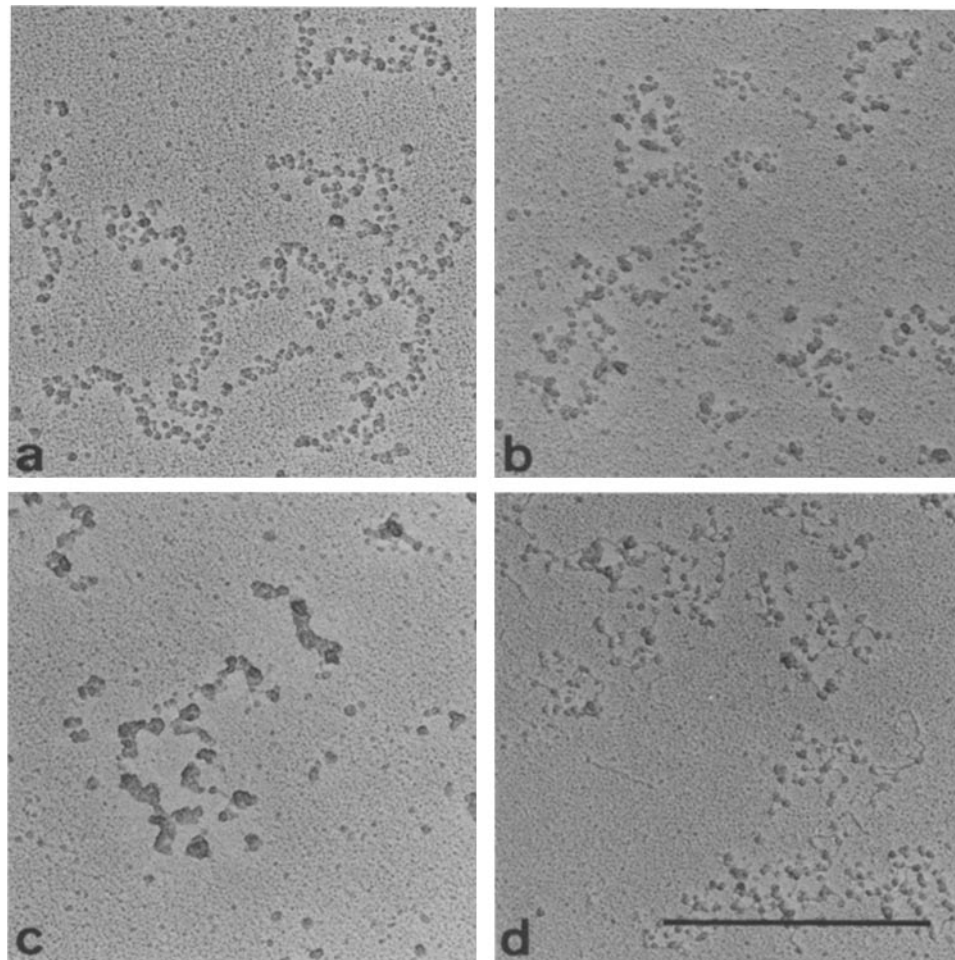


FIGURE 10 (a-c) Dependence of the appearance of chromatin upon the presence of $MgCl_2$. Chromatin at a concentration of ~ 1 mM DNA phosphate was fixed with 0.1% glutaraldehyde at $4^\circ C$ for 15 h in the following solutions: (a) 5 mM TEACl, 0.2 mM EDTA; (b) 5 mM TEACl, 0.2 mM EDTA, 0.2 mM $MgCl_2$; (c) 5 mM TEACl, 0.2 mM EDTA, 0.5 mM $MgCl_2$; (d) H1-depleted chromatin fixed in 5 mM TEACl, 0.2 mM EDTA, 2 mM $MgCl_2$. For adsorption, the fixed samples were diluted to a concentration of $0.02 A_{260}$ U/ml in a spreading buffer containing 5 mM TEACl, 0.2 mM EDTA, and $2 \times 10^{-4}\%$ BAC. Bar, $5,000 \text{ \AA} \times 70,000$.

chloride. A number of previous reports mentioned in the Introduction have described the requirement of divalent cations for a higher order of structural organization of chromatin. Since our experiments in monovalent salt (cf. references 38 and 53) showed that this requirement was not obligatory, we were interested to see the effects of $MgCl_2$ in the fixation buffer. As can be seen in Figs. 10 a-c, a similar folding as described for NaCl and KCl is observed, except that much lower concentrations of $MgCl_2$ are needed than with monovalent cations. This confirms the earlier re-

sults of Finch and Klug (12) who showed that, on adding Mg^{++} , chromatin could fold in solution to form a thick 250-\AA fiber, similar to those that had been observed long before on whole-mount specimens of chromosomes when chelating agents were not used in the preparation (see reference 39). When H1-depleted chromatin is fixed in the presence of $MgCl_2$, the beads-on-a-string type of fiber appears up to 1 mM $MgCl_2$, whereas at 2 mM $MgCl_2$ irregularly folded clumps are observed (Fig. 10 d), similar to H1-depleted chromatin fixed in higher ionic strength of monovalent cations.

SOME TECHNICAL COMMENTS

In the type of specimen preparation employed here, there is an adsorption step, in which the specimen is transferred from the solution to the support film, a washing step for the removal of residual sample and salt, and finally a drying step. Contrast can be achieved in different ways. Contrast enhancement by staining is usually done before drying, when the specimen is tagged with heavy metals (positive staining) or when the specimen is embedded in a layer of heavy metal salt (negative staining). Contrast enhancement may also be done after drying by shadowing with a heavy metal, as in this work. Whatever is done, one has to make sure that the morphology of a labile structure such as that of chromatin is not altered, distorted, and even destroyed during these many steps (54). We now show that a stabilization of the shape in solution is required.

Glutaraldehyde Fixation

When unfixed chromatin is prepared for electron microscopy according to our standard procedure (i.e., adsorbed on carbon-coated grids, washed on water, dehydrated in ethanol, and rotary shadowed), nucleosomes are not observed but instead unravelled filaments appear, presumably DNA with proteins attached (Fig. 11 and Fig. 7*a* and *b*). These filaments tend to curve sharply, often forming small circles, so that the opening of the nucleosomal structures is not simply caused by stretching of the chromatin but might happen during adsorption and/or washing by water. This kind of filament is observed over the whole range of ionic strength investigated and does not reflect what one would expect from the behavior of chromatin in solution (see Fig. 2). We therefore are convinced that the unfixed chromatin loses its morphology under our preparation procedure.

Now, 100-Å fibers of unfixed chromatin adsorbed on carbon films have been observed when negative staining techniques are used (12) (see discussion below). However, as soon as any salt is added to the unfixed preparation, then the 100-Å fiber falls apart into individual nucleosomes (47). To overcome these problems, some form of fixation is required. Glutaraldehyde is an obvious choice, particularly because it is known to cross-link histone proteins irreversibly (6). The glutaraldehyde concentration was kept low (0.1%) to enhance the changes for cross-linking, and preliminary experiments were carried out to determine

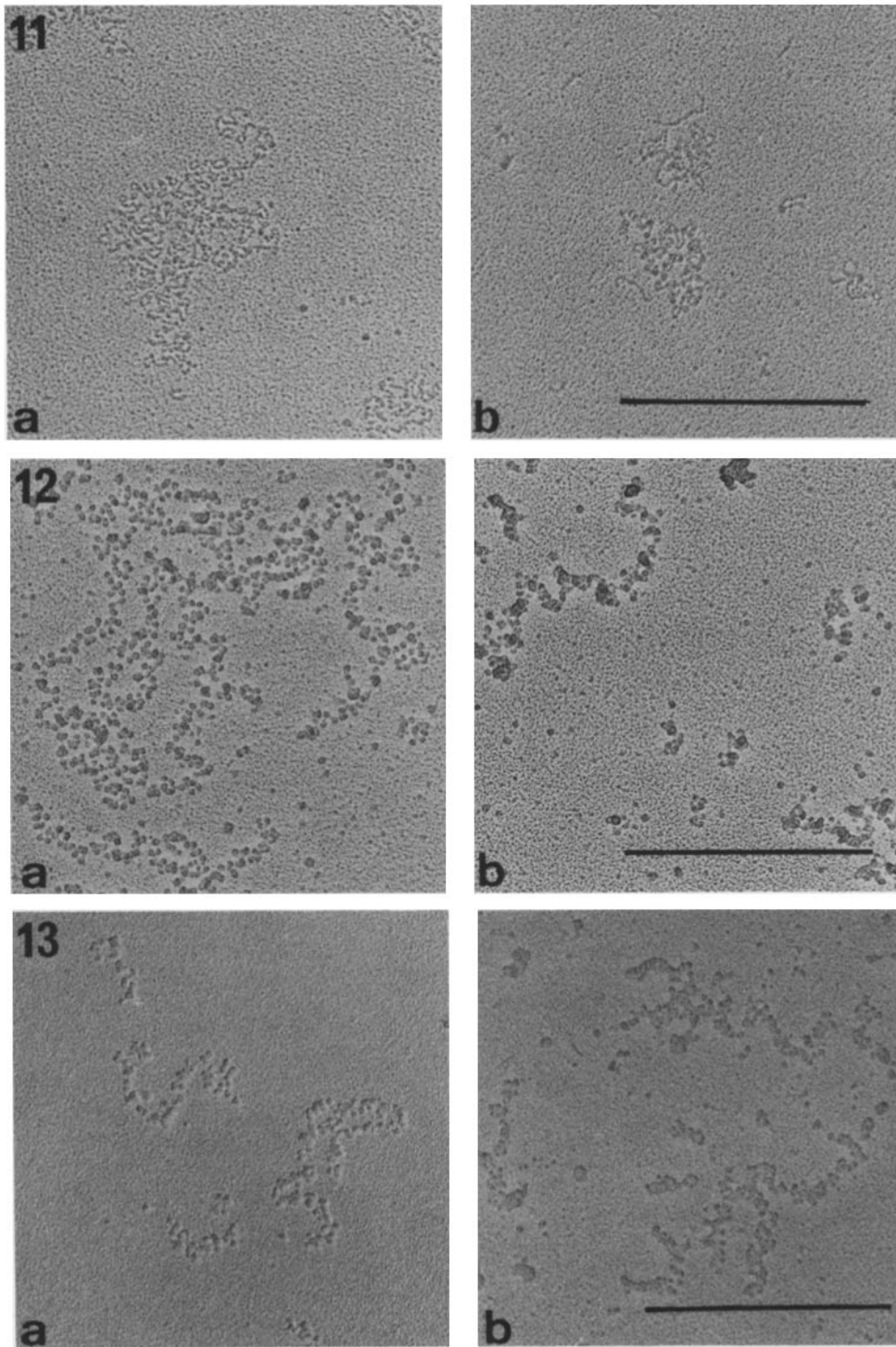
the fixation time necessary to give consistent chromatin forms.

To test for possible fixation artifacts, freshly extracted chromatin was fixed in the same way as for the electron microscope preparations, and its sedimentation coefficient was determined in the analytical ultracentrifuge as a function of ionic strength, in parallel with unfixed control samples (Fig. 2). The average sedimentation value of ~35S in 1 mM TEACl, 0.2 mM EDTA increases to ~80 at 40 mM NaCl. A further increase in ionic strength has little effect on the sedimentation constant. These data are compatible with other observations (38). Since the values determined for fixed and unfixed samples are similar, we assume that the glutaraldehyde fixation does not affect the general shape of chromatin fragments in solution.

Spreading, Washing, and Drying

Table I summarizes the adsorption properties of carbon films in the presence and absence of BAC for fixed and unfixed chromatin in solutions containing different concentrations of NaCl or KCl. At low ionic strength, good adsorption is only obtained if at least $2 \times 10^{-4}\%$ BAC is present (cf. reference 58). However, at 10 mM NaCl or KCl and above, the adsorption is indistinguishable whether BAC is present or not. (For definition of "good" and "bad" adsorption, see legend to Table I). In this range, the appearance of the chromatin is also the same with or without BAC. Therefore, to achieve good adsorption under comparable conditions in the whole range of ionic strengths, BAC was used in all the experiments described in this paper.

Control experiments have been carried out to see whether chromatin fixed in solution at a particular ionic strength can, despite the fixing, undergo further changes in morphology if spread at a different ionic strength and if the grids are further processed as usual by washing on water and dehydrating in ethanol. Spreading from a lower ionic strength gives no change in the appearance compared with spreading at the original, even if reduced to as little as 0.2 mM EDTA (cf. the mixing experiment in Fig. 8). The experiment in the reverse direction was performed using $MgCl_2$. When chromatin, fixed at very low ionic strength, is spread in the presence of 0.2 to 1 mM $MgCl_2$, the fibers appear condensed (Fig. 12), an appearance which does not reflect the structure in the solution in which it is fixed. This shows clearly



that divalent cations can alter the structure of prefixed chromatin.

It has been found that, during washing of specimens of purified DNA, conformational changes may take place.³ To test the washing procedure adopted, specimens of chromatin fixed at very low ionic strength were washed with a solution containing up to 5 mM MgCl₂ and were then directly dehydrated in ethanol without washing first in water. Since only small concentrations of Mg⁺⁺ are required for condensation of chromatin (Fig. 12), it is possible to omit the washing step before drying without the disturbance on subsequent observations produced by salt deposited on the specimen. If the adsorbed chromatin were mobile during the washing, then one would expect to find the condensation of prefixed chromatin spread in MgCl₂ just described. However, no such effect is observed. Therefore, we conclude that the chromatin remains tightly adsorbed to the supporting film and is not mobile under our washing conditions.

It is known (e.g. references 23 and 9) that ethanol dehydration of specimens of purified DNA may induce folded forms. To exclude similar phenomenon with chromatin in the present experiments, samples of the same specimens were compared using ethanol dehydration and freeze-drying (Fig. 13). No significant differences were found, so the ethanol dehydration was adopted as the routine procedure.

Possible "Sliding" of Nucleosomes

Workers in the chromatin field have worried for some time about the possibility of a rearrangement of the original repeating structure, particularly at

elevated salt concentrations (53). Steinmetz et al. (50) have shown that treatment of native chromatin with 0.6 M NaCl results in a reduction of the original repeat length, and suggested that the histones could migrate or slide along the DNA. Such sliding can also be found at lower salt concentrations, particularly in the absence of H1.⁴ We note that some slight, but only slight, sliding might have occurred in our experiments, since occasionally stretches of DNA-like filaments, of length a few hundred angstroms, are seen. These are more evident at the higher salt concentrations (Figs. 5*d* and 10*d*) and are more frequently observed after H1 extraction, which may be related to the destabilization of the nucleosomal structure. Presumably, the rearrangement takes place during the long dialyses used in our preparations, but since these are at 4°C, the effect is minimized. The amount of such DNA-like stretches varies from experiment to experiment, but in all cases they represent only a minute proportion of all the molecules adsorbed to a grid.

DISCUSSION

Condensation of Chromatin

We have seen that, at very low ionic strength and in the absence of divalent cations, chromatin forms a loose fiber consisting of nucleosomes connected by thinner DNA-like filaments, which we have referred to as a nucleosome filament. Upon increasing the ionic strength, a progressive development of superstructure takes place.

ARRANGEMENT OF NUCLEOSOMES IN THE HIGHER-ORDER STRUCTURES: The systematic observations allow us to reason how the nu-

³ Koller, Th. Unpublished results.

⁴ Chambon, P., and R. D. Kornberg. Private communications.

FIGURE 11 Unfixed chromatin spread in (a) 5 mM TEACl, 0.2 mM EDTA, and $2 \times 10^{-4}\%$ BAC (cf. Fig. 3*b*); (b) the same as a but with 100 mM NaCl (cf. Fig. 3*f*). Bar, 5,000 Å. $\times 70,000$.

FIGURE 12 Influence of the spreading buffer on chromatin fixed at low ionic strength. (a) Chromatin fixed with 0.1% glutaraldehyde in 5 mM TEACl and 0.2 mM EDTA and spread in the same buffer plus $2 \times 10^{-4}\%$ BAC. (b) The same as in a but 1 mM MgCl₂ was added to the spreading buffer. Bar, 5,000 Å. $\times 70,000$.

FIGURE 13 Specimen preparation by freeze-drying. (a) H-1 containing chromatin was fixed with 0.1% glutaraldehyde in 5 mM TEACl, pH 7, and 10 mM NaCl, adsorbed on grid, washed for 10 min with distilled water, then freeze-dried as described in "Methods." (b) Same procedure as in Fig. 13*a*, except that the grids were not washed on water before freeze-drying. Note: The salt residue blurs the fine-structural details but the overall appearance of the fibers is the same as given by the standard procedure (compare with Fig. 3*c*). Bars, 5,000 Å. $\times 70,000$.

TABLE I
Adsorption of Chromatin to Carbon Support Films: The Influence of Ionic Strength and the Presence of BAC in the Spreading Buffer

TEACl, pH 7.0 (mM)		1	5	5	5	5	5
EDTA (mM)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
NaCl (mM)				10	40	60	100
BAC	+	+	+	+	+	+	+
no BAC	-	-	-	+	+	+	+

The data given are for glutaraldehyde-fixed and unfixed samples. + Denotes "good" adsorption; - denotes "bad" adsorption. "Good" adsorption is defined according to the following criteria: (a) at a chromatin concentration corresponding to $A_{260} = 0.02$, the whole grid is uniformly covered with chromatin in fibers as in Figs. 3, 5, 8, 9, and 11. (b) The fibers do not show stretched or streaked regions which would imply insufficient adhesion points between sample and carbon film.

cleosomes might be arranged in the thick fibers, even though their relative positions and orientations are no longer resolved with accuracy in electron microscopy. In developing this picture (Fig. 14), we keep in mind that the fixative used (glutaraldehyde) cross-links protein to protein (6).

Since H1-containing chromatin fixed at very low ionic strength shows a nucleosome filament in which the nucleosomes are well separated, this morphology indicates that there is no stable cross-linking of neighboring nucleosomes to each other and that the nucleosomes in solution must be separated over a distance longer than the glutaraldehyde molecules. At somewhat higher ionic strength, this nucleosome filament folds up to form a fiber with the appearance of nucleosomes arranged in a close zigzag, the internucleosomal DNA being no longer visible. This fiber represents the first higher-order structural arrangement of nucleosomes and is the key to the interpretation of the more condensed higher-order structures. First, adjacent nucleosomes along a helical turn are stably cross-linked: this is shown by the fact that if chromatin fixed in 5 mM TEACl and 0.2 mM EDTA is spread from a solution of lower ionic strength (0.2 mM EDTA), it does not open into a nucleosome filament. The nucleosomes are round in shape and remain in contact. The most reasonable explanation is that the nucleosomes were cross-linked edge to edge at one point, maintaining the structure of the close zigzag. Second, histone H1 is involved in these contacts, since H1-depleted chromatin does not show fibers with nucleosomes in close (zigzag) contacts. Third, we have deduced the location of H1 on the nucleosome at the site where the DNA enters and leaves the nucleosome (see Results and below). Fourth, from the development of the open zigzag into a

close zigzag in H1-containing chromatin, it follows that the continuity of the DNA in this structure is across the rows of the zigzag, i.e., along the turns of the equivalent helix in solution. Fifth, since we have not observed nucleosomes stacked face-to-face on each other as found in the "nucleofilament" and in crystals of core particles (12, 13), we conclude that there is not stable face-to-face cross-linking between the core histones of successive nucleosomes along the DNA.

From all these arguments, we conclude that in solution the successive nucleosomes touch each other and are stably cross-linked by glutaraldehyde close to the site of the incoming and outgoing DNA, presumably by H1 to H1 contacts. The planes of the platysomes make an angle with each other that makes face-to-face cross-linking unlikely. It is hard to imagine any other arrangement to explain the results other than this type of helical structure.

The parameters of the helix such as the pitch, and the number of nucleosomes per turn can be inferred from the way in which the arrangement of nucleosomes changes and their visibility under identical shadowing conditions decreases in the series of micrographs at increasing ionic strength. The flat fiber with the predominant zigzag appearance is, as already stated, compatible with a helical structure with two to three nucleosomes per turn and with a pitch of about one nucleosomal diameter. Nucleosomes are less and less resolved within the fibers as the ionic strength increases, but the spacing of the cross-striations that indicate helical coils remains roughly constant (~100–150 Å or one nucleosomal diameter). We thus think that the chromatin is organized in a helical structure with a constant pitch of about one nucleosomal diameter (cf. the x-ray results of Sperling and Klug

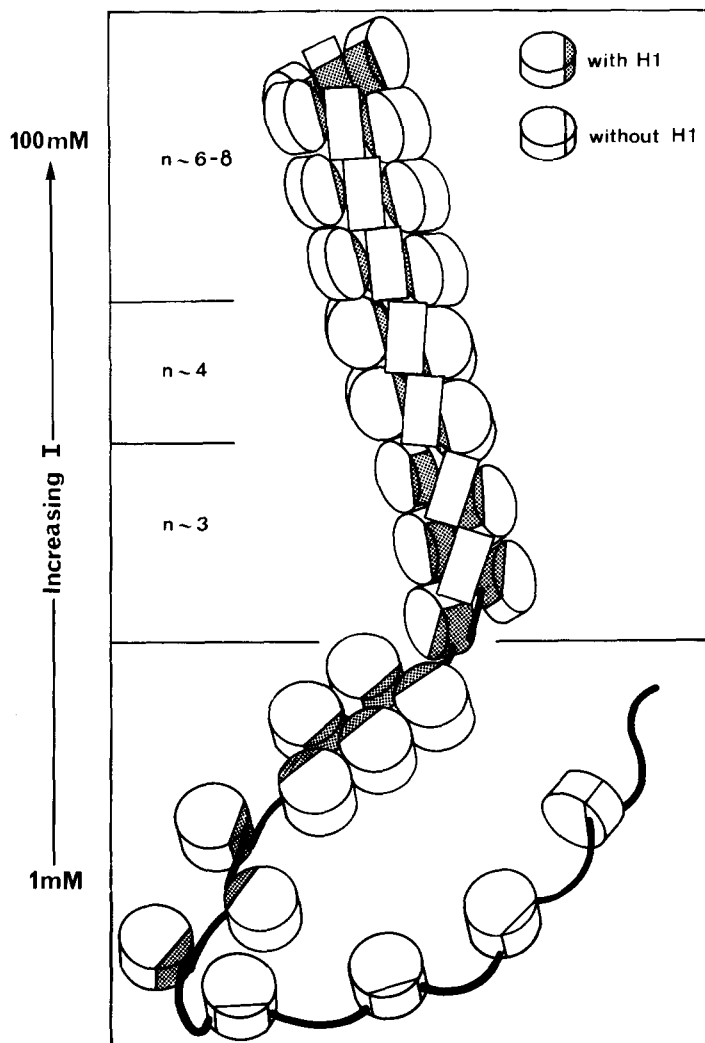


FIGURE 14 Idealized drawing of helical superstructures formed by chromatin containing H1 with increasing ionic strength. The open zigzag of nucleosomes (bottom left) closes up to form helices with increasing numbers of nucleosomes per turn (n). For simplicity the helices are drawn as regular, but the observations show them to be rather irregular, as might be expected for a structure in which the main contacts are in a central flexible core. The structures may be said to be organized, but not perfectly regular (see text). When H1 is absent (pictured at bottom right), no zigzags or definite higher-order structures are found.

(48) and we interpret the change in the morphological appearance of the chromatin fibers with increasing ionic strength as an increase in the numbers of nucleosomes per turn as depicted in idealized form in Fig. 14 without taking the irregular nature of the native chromatin fiber into account. This leads to only a small increase in the diameter and a decrease in the pitch angle. The diameters of the chromatin fibers are between 200

and 300 Å.

With the increasing number of nucleosomes per turn, the face-to-face angle between platysomes decreases as well. This explains the difficulty of depositing platinum between the nucleosomes and to resolve them within the thick fibers, at 60 mM NaCl and above where about six nucleosomes per turn can be estimated (see also reference 12). Moreover, it explains the fact that the reaction rate

of the glutaraldehyde cross-linking of core histones increases with the ionic strength, whereas the rate of formaldehyde fixation (which does not cross-link histones) remains constant (6). The proximity of the faces of adjacent nucleosomes at high ionic strength increases the chances of cross-linking.

In the model presented here, adjacent turns of the helix touch each other, presumably involving DNA-to-DNA contact, and this may explain why divalent cations are particularly effective in stabilizing such higher-order structures. The unraveling of higher-order structures to the nucleosome filament at very low ionic strength is thus to be understood in terms of electrostatic repulsion between charges on the chromatin DNA that leads to a separation of the nucleosomes in solution.

CORRELATION WITH PHYSICOCHEMICAL OBSERVATIONS: It is known that with increasing ionic strength the sedimentation coefficient and turbidity of chromatin increases (35, 1, 25). These measurements indicate an increasing compaction, but unfortunately the data available do not lend themselves to quantitative analysis. Data which, however, can be interpreted come from experiments on the scattering of x-rays or light by chromatin in solution.

A study by low-angle x-ray scattering of solutions of chromatin in 0.2 mM EDTA has shown it to be an extended structure with a mass per unit length of $\sim 2,300$ daltons per \AA (or, in other words, about one nucleosome per 100\AA) and an axial radius of gyration of 38\AA (46). These values are quite consistent with the nucleosome filament we have observed at very low ionic strength. Although the nucleosomes are not touching, they are connected by short lengths of DNA which do not lie parallel to the length of the filament, so that the filament is somewhere between one and two nucleosomes wide on the average.

At higher ionic strengths, some light-scattering data are now available for chromatin fragments of different lengths ("polynucleosomes"). Campbell et al. (5) measured the radius of gyration (R_g), which reflects the maximum extension of a fragment as a function both of size of fragment and of ionic strength. R_g is found to increase linearly with the size of the fragment, showing that the shapes adopted are essentially linear extended ones. Campbell et al. (5) interpreted their data in terms of a number of specific models. One can consider their data in model-independent terms by deriving from them the axial fiber length per nucleosome (= translation distance h , see Table II). If we take

TABLE II
Values of h , the Axial Translation between
Successive Nucleosomes

	h	n ($p = 110 \text{\AA}$)
	\AA	
10 mM Tris, 0.7 mM EDTA	34	~ 3
40 mM NaCl, 10 mM Tris, 0.7 mM EDTA	24	~ 5
60 mM NaCl, 10 mM Tris, 0.7 mM EDTA	20	~ 6

Original data from Campbell et al. (5). The calculation of h can be illustrated as follows: $h = p/n$; at 10 mM Tris, Fig. 3 in reference 5 gives $h = 210 \text{\AA}/6 N = 35 \text{\AA}/N$, Fig. 4 gives $440 \text{\AA}/13 N = 34 \text{\AA}/N$, Fig. 5 gives $300 \text{\AA}/9 N = 33 \text{\AA}/N$, making a mean of $34 \text{\AA}/N$ for model-independent value of h .

n = No. of nucleosomes per turn.

p = Pitch distance.

N = nucleosome.

the chromatin fibers to be helical at all ionic strengths, as we have observed, and assume that the nucleosomes tend to lie with their planes parallel to the fiber axis and that neighboring turns of the helix tend to touch, as we have also observed, then the pitch of the helix in all cases would be equal to the diameter of the nucleosomes, namely 110\AA (13). With this value, one can calculate n , the number of nucleosomes per turn of the helix (Table II). If the helical pitch were greater, n would be correspondingly greater. We see that n increases with ionic strength and that the trend of values agrees well with the electron microscope observations summarized in Fig. 14. At the lowest salt concentration the number of units per turn is ~ 3 , agreeing well with the fibers observed by Thoma and Koller (53) and repeated in this work. At the highest salt concentration, as already remarked by Campbell et al. (5), the light-scattering data agree with the solenoidal model proposed by Finch and Klug (12) and drawn in idealized form in Fig. 14. The value of $n = 6$ for the solenoid must be regarded as approximate.

Finally, the solenoidal model provides an explanation for the origin of the x-ray reflexion at 110\AA long known to be given by chromatin. It would correspond to the spacing between the turns of a solenoid rather than to the interval between nucleosomes along the direction of the 100-\AA filament (12). X-ray studies by Sperling and Klug (48) on chromatin in the filamentous and thick fiber states have confirmed this: the 110\AA reflex-

ion is lost in the extended state. The present electron microscope study shows that, in the extended nucleosome filament, nucleosomes are not in contact and therefore not 110 Å apart. Again, Sperling and Klug (48) found that the 110-Å reflexion is lost in H1-depleted chromatin under conditions in which it is present in complete chromatin. The x-ray and electron microscope observations are therefore consistent.

QUESTIONS OF NUCLEOFILAMENTS, SUPERBEADS, AND PAIRS OF 100-Å FIBERS: Finch and Klug (12), examining unfixed chromatin at 0.2 mM EDTA by negative staining, found 100-Å fibers in which the nucleosomes were not resolved, and defined the "nucleofilament" as one in which adjacent nucleosomes were in close contact. However, Hozier et al. (19) have published micrographs of negatively stained, fixed material which show a beaded chain of nucleosomes. The fibers observed by Finch and Klug (12) may have arisen through use of relatively high concentrations of chromatin, and some of them may not have been completely adsorbed to the carbon-coated grid and therefore could be stabilized by the uranyl acetate employed in the negative staining, to form structures rather like the columns found in the crystals of nucleosome cores (13). In such columns the isolated nucleosomes stack face-to-face, and our results on fixed material suggest that this is not characteristic of native chromatin in solution at very low ionic strengths, where the contact between nucleosomes appears to be edge-to-edge. To avoid any ambiguity, we have therefore used the term "nucleosome filament" in this paper.

Hozier et al. (19), examining chromatin fixed at ~40 mM ionic strength, found discontinuous thick fibers, which sometimes appeared to consist of globules having a periodicity of ~200–300 Å along the fiber length (see also reference 51). They termed these globules "superbeads," postulated to contain about eight nucleosomes arranged in a regular superstructure. Worcel (60) has proposed that the superbeads arise from a discontinuous solenoid, regularly interrupted every two turns.

As described above, we have also observed this type of globular appearance in some fibers but never consistently regular enough to warrant a separate description. Since we have observed the onset of helical condensation at ~20 mM NaCl and its completion at ~60 mM NaCl, an ionic strength of 40 mM NaCl represents the intermediate region. In this range, we have often seen

helical structures which tend to fall apart irregularly into individual turns or pair of turns (Fig. 4 *d* and *e*). We therefore think that the appearance of "superbeads" arises as a consequence of a local association, preserved by fixation, of a turn or of neighboring turns of a flickering, helical structure which has only a weak attraction between turns. At a higher ionic strength, the globules are much less evident as separate entities, although the 250-Å fibers are still irregular in diameter, as remarked by Finch and Klug (12), or "knobbly" as stated by Hozier et al. (19), and as seen in Figs. 3 and 4.

Hozier et al. (19) have suggested that the difference between the solenoidal and superbead appearances arises because of the mode of preparation, the former being produced when the nuclei, after the initial micrococcal nuclease digestion, are lysed into 0.2 mM EDTA, and the latter when the lysis is into salt. We do not agree, since we have found the same series of ionic-strength-dependent structures, whichever conditions are used for the original preparations.

One further argument used by Hozier et al. (19) in the support of the existence of superbeads was the pause they observed at 40S in sedimentation patterns of micrococcal nuclease digests of nuclear chromatin. However, if the chromatin is condensed into a helical structure, then the kinetics of digestion is very likely to be nonrandom since the enzyme, having acted, can just as well jump to the next turn of a helix as further along on the same turn. The population of fragments will therefore contain a preferred proportion corresponding roughly to the size of a turn or two turns of the helix. If this interpretation is correct, then the nuclease digestion data of Renz et al. (38) and Strätling et al. (51) would indicate about seven to eight nucleosomes per turn of helix.

While discussing interpretations of electron micrographs of chromatin different from ours, it is pertinent to comment on the possible origin of the claim, widely scattered through the literature (e.g. reference 40), that the 200-Å fiber consists of two 100-Å fibers side by side. While not doubting the possible existence of such structures in certain cases (e.g., in stretched loops), we recall that chromatin observed on the grid at ~10 mM NaCl has the form of a close zigzag (Fig. 4 *b*). Since the linker DNA cannot be seen here as it can in the open zigzag forms (Fig. 4 *a*), this appearance could well be misinterpreted as two parallel 100-Å strands, rather than as a single helix with about two nucleosomes per turn, i.e., zigzag as we have

done. Particularly, in freeze-dried and shadowed specimens (Fig. 13), chromatin at this ionic strength can appear as two ridges ~ 100 Å apart separated by a valley, strengthening the impression of two parallel fibers.

Condensation of H1-depleted Chromatin

Histone H1 has been shown to be necessary for the formation of intermediate higher-order structures of soluble chromatin (53) and also for that of the thick 250-Å fibers (12, 38). Brasch (4) has shown that the thick fibers that can be seen in sectioned and stained nuclei (cf. reference 7) are irretrievably lost when H1 is removed. The implication of H1 in establishing or maintaining higher-order structures is thus clear. In this study we have confirmed our earlier observations on soluble chromatin and extended them to give a general view of the condensation of H1-depleted chromatin. The detailed comparison between the appearances of this H1-depleted chromatin and chromatin containing H1 gives us insight into the particular way in which H1 is involved in the organization of the superstructure.

We have shown that H1-depleted chromatin condenses upon increasing ionic strength, but that it does not lead to the characteristic superstructure formed by complete chromatin. The hydrodynamic data bear this out. On increasing ionic strength, the sedimentation constant increases and the viscosity decreases (25) but the rate of increase of sedimentation constant is much greater for H1-containing chromatin than for H1-depleted chromatin (Fig. 4 in reference 38). In the latter case, the condensation may simply be a direct effect of ionic strength on the extension of a charged polyelectrolyte. Vollenweider et al. (58) have shown that DNA itself at higher ionic strengths forms some kind of superstructure, and the exposed linker DNA in the H1-depleted chromatin may be affected in the same way. The steeper rise in sedimentation constant in the case of H1-containing chromatin suggests that an additional factor is at work, as an effect either on the conformation of the H1 molecule or on its intermolecular interactions. This is further discussed below.

Our observations at low and very low ionic strengths point to the reason why H1 has to be present for the formation of more ordered superstructure at higher ionic strengths, since they show that H1 stabilizes the nucleosome itself.

At ionic strengths of 5–10 mM NaCl, distinct nucleosomes are evident in H1-depleted chromatin,

but the chain of nucleosomes has a beads-on-a-string appearance in which the DNA does not enter and leave the nucleosome on the same side, as is found in complete chromatin (Fig. 7). (A similar morphology of the filament can also be found in H1-containing chromatin when, during adsorption to the grid, it is sheared or pulled out or when it is released from nuclei under sudden lysis [e.g. reference 31, and many references thereafter.]) The loss of structure is not caused by a direct effect on the structure of the nucleosome core since the sedimentation and diffusion constants of isolated cores remain constant from an ionic strength of 100 mM down to 10 mM (16). The simplest interpretation of the electron microscope observations is therefore that H1 is somehow involved in stabilizing the correct entry and exit of DNA to and from the nucleosome core. We shall return to this point in more detail in the next subsection.

At still lower ionic strengths (<1 mM TEAC1), nucleosomes are lost in H1-depleted chromatin and instead there are seen extended, irregular filaments, with an occasional beadlike object still present here and there. This is consistent with an opening up of the nucleosome core since the sedimentation constant of isolated cores changes abruptly below an ionic strength of 1 mM (16). These filaments are thicker than naked DNA and are interpreted to be DNA coated with or attached to protein. A similar linearization or opening out of nucleosome core particles was also obtained by breaking hydrophobic bonds with urea (32) or by thermal denaturation (41). Therefore, we interpret the unraveling of the nucleosomal structure at very low ionic strength as an effect of electrostatic repulsion which overcomes the hydrophobic bonding between the core histone proteins.

QUESTION OF UNRAVELED NUCLEOSOMES IN CHROMATIN: Extended chromatin fibers, similar to those we have observed on H1-depleted chromatin below 1 mM salt, were observed at very low ionic strength by Oudet et al. (34) but with cellular chromatin presumably containing H1. This would seem to conflict with our observations on H1-containing chromatin where the nucleosomes remain intact under such similar conditions. However, the results of Oudet et al. (34) were obtained in experiments where either no fixative or formaldehyde was used. Formaldehyde is known to cross-link proteins to the DNA but not to cross-link the histone proteins themselves (6). A formaldehyde-fixed nucleosome may there-

fore still unravel during adsorption at very low ionic strength. However, with glutaraldehyde fixation it is possible to tell whether nucleosomes were present or not in solution, as in our experiments described above, where a difference is found between H1-depleted chromatin at 5 mM salt, where nucleosomes are observed, and H1-depleted chromatin at 1 mM, where they are not (summarized in Fig. 7c and d and Fig. 9). Therefore, our electron microscopical evidence is that H1-containing chromatin does not unravel at ionic strengths below 1 mM salt but only does so when the H1 is removed. This points to the role of H1 in stabilizing the nucleosome at very low ionic strength. We do not know the factors involved in this stabilization of the nucleosomal structure by H1. One contribution could simply be charge neutralization by histone H1. The results of Oudet et al. (34) are then perhaps to be understood in terms of the dynamic equilibrium suggested by them between the open and beaded forms of the chromatin filament. Fixation by formaldehyde may still permit the opening of a nucleosome, whereas glutaraldehyde fixation would prevent any displacement of the equilibrium towards the unraveled form.

CONCLUSIONS

Localization of Histone H1

We have seen that there is a clear difference in the appearance of nucleosomes in chromatin containing H1, where the linker DNA enters and leaves on the same side of the nucleosome, and in chromatin depleted of H1, where the entrance and exit positions are more or less on opposite sides, and in any case more randomly located. We have also seen that H1 stabilizes the nucleosomal structure and prevents its unraveling into a linearized structure at very low ionic strength. We therefore concluded that H1 must be located at, or close to, the region where DNA enters and leaves the nucleosome.

How does this conclusion relate to what is known about the internal structure of the nucleosome (reviewed in references 21 and 10)? The nucleosome has been defined as the repeating subunit of chromatin produced by brief digestion with micrococcal nuclease: it contains all five histones H1, H2A, H2B, H3, and H4 in molar amounts of 1:2:2:2:2 and, in rat liver, ~200 bp of DNA, though this number varies between different species and tissues. Recent data obtained by

Hayashi et al. (17) confirm earlier data obtained by Varshavsky et al. (56) that demonstrate that there is only one molecule of H1 per nucleosome. Further digestion with micrococcal nuclease leads to a stable limit particle, called the core particle, containing ~140 bp. More recent data give a value of 146 ± 1 (28). The core particle contains two pairs of each of the four core histones H2A, H2B, H3, and H4, but no H1. For this reason, it has been suggested that histone H1 is associated with the linker DNA of length 60 (= 200–140) bp between nucleosome cores (in rat liver).

This picture of core and linker derived from enzymatic studies may not represent the true structural position. During the exolytic degradation of the nucleosome to the core particle, there is a pause in the digestion at 160 bp (now 166), and it is during the step from "160" to "140" bp that H1 is released (e.g. reference 29). Moreover, it has been found from x-ray crystallographic studies that the nucleosome core is a flat disk-shaped particle, the "platosome," containing about one and three-fourths turns of DNA arranged in a superhelix of pitch ~27 Å. It has therefore been suggested (13, 20) that the 166 bp particle contains a full two turns of the DNA superhelix which brings the two ends of the DNA close together (Fig. 15; Fig. 12 in reference 14). Since the single H1 molecule is released during the passage from 166 to 146 bp, it is clear that H1, or part of it, is associated with the ends of the DNA on the complete two-turn particle, as depicted in Fig. 15.

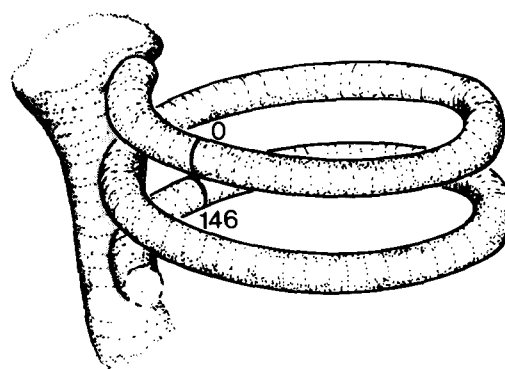


FIGURE 15 Diagram to show how H1 "seals off" the nucleosome. The 146 bp nucleosome core particle (marked by bars) is extended by 10 bp at each end to form a full two-turn particle of 166 bp. H1, or strictly part of it (shaded, shown schematically), is attached to the sites on the side of the two-turn particle at which DNA enters and leaves.

Returning to our electron microscope observations on the nucleosome, one can therefore say that H1 stabilizes, or as it were, seals off a two-turn DNA nucleosome. On this view, and using the newly determined values for the DNA content of the core, the linker DNA should perhaps be redefined according to the following structurally symbolic equation:

200 bp nucleosome repeat (in rat liver)	=	166 bp complete two-turn particle	+	34 bp linker DNA
variable between species and tissues		invariant		variable between species and tissues

rather than $200 = 140 + 60$ as hitherto.

It would now also follow that histone H1 is not simply bound linearly along the DNA, but that H1, or part of it, is designed to interact with a unique three-dimensional arrangement of DNA double helices, namely that on the side of the nucleosome where there are three distinct segments of DNA, $\sim 27 \text{ \AA}$ apart (stippled area in Fig. 15).

Indeed, there is some physicochemical evidence that H1 is designed for just such a role, i.e., that it contains at least two sites for binding DNA lengths which are roughly parallel and close together. Singer and Singer (44) have found that H1, at relatively low H1:DNA ratios, binds strongly to circular, superhelical DNA but not to circular, relaxed DNA. Bina-Stein and Singer (2) went on to show that H1 does not introduce any superhelical twists in the DNA, but stabilizes the preexisting superhelical turns. Singer suggests that H1 is binding to the ends of superhelical loops, but there is another possibility. Since, under the prevailing salt conditions, the superhelical DNA would be in the interwound superhelical form (15, 58) in which lengths of DNA come into close juxtaposition, a possible explanation of Singer's result is that H1 is straddling two roughly parallel lengths of DNA close together, rather as it would be doing on the side of the nucleosome particle pictured in Fig. 15. On the other hand, relaxed circular DNA does not have lengths of DNA arranged side by side so that the special situation for strong H1 binding is not present. Weaker binding at a single site is still possible, however, so that with relaxed DNA one would have to go to higher H1/DNA ratios to

obtain significant binding, as was observed by Singer and Singer (44).

A Limiting DNA Repeat in Chromatin or a Minimal Nucleosome?

Values for the DNA content of a nucleosome have been obtained for a wide range of cell types. The most frequent value is ~ 200 bp, but the variation is remarkable (see reference 21). On the other hand, the nucleosome core particle containing "140" bp and the histone octamer seem to be universal. In the light of the model just presented, we would like to suggest that the basic structural element common to all chromatin is a larger particle, containing 166 bp of DNA organized in two full superhelical turns, the histone octamer and one molecule of H1 (Fig. 15). This particle would represent the minimal nucleosome, and would account for the fact that the lowest values reported for the DNA repeat in chromatin are ~ 160 – 170 bp.

The hypothesis (13) that the 166 bp particle represents the 146 bp particle extended by 10 bp at each end, rather than, say, by 20 bp at one end can be tested by isolating "160" bp particles and seeing how the characteristic enzyme digestion pattern of the "140" bp core particle (e.g. references 42 and 27) has changed. This has been done by Simpson (43) using DNase I, and by Lutter (27, and footnote 5) using both DNase I and DNase II. The results confirm the hypothesis.

Direct evidence for a stable particle containing 166 bp of DNA and H1 has recently been obtained by Prunell and Kornberg (36, and footnote 6). Beginning with rat liver nucleosome monomers containing 193 bp, and taking precautions not to lose H1, they have found that such particles "trimmed" using a combination of exonuclease III and spleen phosphodiesterase I (36) reduce to a particle containing 166 ± 6 bp of DNA on which H1 is still present. Similar experiments using nucleosome dimers give a value for the average length of the linker DNA, and the results show that no significant number of monomers can be present with a DNA content < 166 bp.

Organization of Higher-Order Structures by Histone H1

The picture we have built up of the location and

⁵ Lutter, L. C. Private communication.

⁶ Prunell, A., and R. D. Kornberg. Private communication.

role of H1 on the nucleosome can be extended to make a hypothesis on the way in which H1 is involved in the organization of the 250-Å helical fibers found at moderate and high ionic strengths. First, we have shown that H1 is necessary for the condensation of chromatin into these definite superstructures. (H1 alone may not, of course, be sufficient, since, when H1 is removed or added back in the experiments described, there are always nonhistone proteins accompanying it. This remains a problem for the future). Second, we have argued that, in the helical superstructures of pitch ~ 110 Å, the nucleosomes (platysomes) are arranged with their planes oriented more or less parallel to the axis of the helix rather than perpendicular to it (Fig. 14). This means that the DNA entrance and exit points on the nucleosome, with which the H1 molecule is associated, are close to those on the adjoining nucleosomes on either side of it on the same turn of the helix. H1 molecules would thus be located on the inside of the helix (or solenoid) of nucleosomes, as proposed earlier, (12), and be in a position to touch each other. This explains the formation of homopolymers of H1 by various types of cross-linking, both in nuclei (30) and in chromatin at high salt (6, 55). A highly detailed model of this kind has been worked out by Worcel and Benyajati (59), but this model differs from ours in the relation of H1 to the nucleosome and to the linker DNA. In any case, the basic assumption of Worcel's model that the curvature of the DNA distributes itself so as to be constant over nucleosome core and linker regions is invalidated by the observation of Finch and Klug (12, see also reference 48) that 300-Å solenoidal fibers can be formed out of isolated nucleosomes. The continuity of the DNA is thus not essential for the formation of the helical superstructure.

The important point is that it would seem to be the aggregation of H1 into a helical structure which accompanies, and indeed, may control, the formation of the chromatin superstructure. The flexibility of the thick 250-Å fibers, and the irregularities in diameter and pitch seen in the electron micrographs, might arise precisely because the essential contacts are those near the central poly-H1 aggregate, rather than those between the faces of the nucleosomes which could be, as it were, merely carried along on the poly-H1 core. From our series of electron micrographs of cross-linked chromatin, it would rather seem that the main direction of connections in the H1 polymers is

along the superhelical turns rather than between them. A helical structure of this kind does not require all the nucleosomes to be in strictly equivalent positions: it is organized but not perfectly regular. Moreover, since its main contacts are in the center, it would be easily deformed and so be able to fold back further on itself, as would be required to give still more condensed states of chromatin. Our observation that there is no consistent left or right handedness to the helical fiber also fits a model in which there is no requirement for strict equivalence between repeating units, and in which all that is determined is the approximate curvature and pitch of the helix.

Our observations are not on a fine enough scale to show the disposition of the "true" linker DNA between the H1-sealed, two-turn nucleosomes. Some of it may be associated with other parts of H1. There is not serious structural difficulty about fitting in linker DNA, which may be quite variable in length, into a structure which is not highly regular. The "extra" DNA may be expelled into the interior. A further detailed problem for the future is that of understanding how only one H1 molecule is attached to a two-turn nucleosome with (putative) dyad symmetry, so as to break the symmetry and make (putative) polar higher-order structure.

If the polymerization of H1 is a key to the formation of superstructure, how then is the condensation induced by salt? To try to understand any specific effects, other than the general reduction of electrostatic repulsion which exists also for H1-depleted chromatin as discussed above, one turns to the properties of H1. First, H1 seems to undergo a large change in conformation at ~ 20 mM salt (45), and there is also some evidence for a cooperative interaction with DNA above this ionic strength (37). This can perhaps be related to our observations, because it is at about this ionic strength that the first true three-dimensional structure appears (Figs. 3 and 4) in which a given nucleosome is in contact with more than just the two nucleosomes to which it is joined on either side by the linker DNA. It is not possible to say whether the salt has a direct effect on the conformation of H1 or, rather, on the special H1-multiple site DNA complex described above. It is noteworthy that, whereas other purified histones have a notorious tendency to self-aggregation, this is not the case with H1, even in 0.1 M NaCl (49). If this is due to charge repulsion, then the neutralization of charge on the H1 by the interaction with DNA

could make aggregation possible. Moreover, on binding to the special site on the nucleosome, H1 might undergo a conformational change which enables it to form H1 polymers. There is, of course, always the further possibility that an H1 molecule on one turn of the solenoid can then also interact with the DNA (or H1) on the nucleosomes of the next turn above or below. It is to be hoped that this possibility can be checked by other types of cross-linking experiments. Of course, electron microscopy by itself cannot prove a structure in solution, but with the precautions taken the morphology seen can reflect the structures present in solution, and so allow the building a model of the structure in solution. Hydrodynamic studies under comparable conditions are in progress.

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