HIGHER-ORDER STRUCTURE OF CHROMATIN FROM RESTING CELLS

I. ELECTRON MICROSCOPY OF CHROMATIN FROM CALF THYMUS

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SUMMARY
Extremely large domains of the genome of resting cells (calf thymus) have been visualized in the electron microscope by combining mild extraction procedures with a non-artifactual method of mounting the sample (the phospholipid monolayer technique). The observed chromatin strands, free from distortion, reach contour lengths up to 60 μm.

After lysis of the nuclei, four classes of fibres may be identified on the basis of their diameters (30, 24, 18 and 11 nm, respectively). The morphology of giant chromatin strands is strikingly regular; long trains of equally sized, arc-shaped segments are observed, their length being, in many cases, multiples of a fixed value. The inflection points delimiting contiguous segments are often associated with laminar fragments of the nuclear envelope or, less frequently, linked to fibrillar elements. It appears that higher-order structures of chromatin in resting cells conform, to a large extent, to a so-called 'drapery-like' model, according to which a continuous strand runs between contiguous anchorage sites placed on the nuclear envelope. Because of the presence of regularly spaced inflection points, this organization is much more ordered than expected.

Spontaneous unwinding of the fibres at low ionic strength, limited nuclease digestion, and relaxation in the presence of ethidium bromide, have been used as probes of the conformation. All these experiments rule out its identification with a single-strand helix. The final ordered state is attained by folding the basic 11 nm strand and by winding up this configuration on itself. This leads to a coiled-coil or 'rope-like' model. The 11 nm strand is 'punctuated' by sharp kinks. Roughly, it may be assimilated to a chain of semirigid, freely joined elements. As a consequence, local flexibility is greatly enhanced, so allowing the assembly mode described.

INTRODUCTION
The notion of chromatin condensation has become very popular for workers interested in the field of physical chemistry of biopolymers, following the elegant visualization of this phenomenon presented by Finch & Klug (1976) and, subsequently, by Thoma, Koller & Klug (1979). Both the v bodies (Olins & Olins, 1974) or nucleosomes (Oudet, Gross-Bellard & Chambon, 1975) and the 'linker' DNA are clearly discernible as a beaded filament; compaction of this structure is fully achieved by raising the monovalent salt concentration to 60 mM (Thoma et al. 1979). The very exciting consequence of these results is that they mimic, although at ionic strengths well below the physiological range, the two abrupt uncondensed--condensed state transitions observed for chromatin during the cell cycle (Nicolini, 1980). Hence, theories relating condensation phenomena to the polyelectrolyte nature of the nuclear
components have been put forward (Manning, 1979; Belmont & Nicolini, 1981).

A plethora of models for the structure of the condensed state has rapidly become available. We shall limit ourselves to recalling the main features of some prototypes, disregarding their subsequent variations. Early work by Finch & Klug (1976) and Nicolini & Kendall (1977) interpreted the 25–30 nm thick fibre in terms of a flat helix (solenoid) or of a multiparameter supercoil, respectively; recently, Worcel, Strogatz & Riley (1981) proposed, on the basis of subtle topological considerations, a zig-zag twisted ribbon. Opposed to these helical structures, we find different 'superbead' models (Hozier, Nehls & Renz, 1977; Stratling, Müller & Zentgraf, 1978), built up by clustering the nucleosomes according to a spherical array. What is observed in the electron microscope by freeze-fracture methods (Lepault, Bram, Escaig & Wray, 1980) is at variance. The basic conformation of the polynucleosomal fibre is a loose 25 nm diameter supercoil, which winds up on itself, or doubles to form a 50 nm thick strand.

This state of affairs should not be too surprising for the electron microscopist expert in the field; he aims at portraying the architecture of large genome domains; but, in addition to preparative difficulties, the risk of artifacts increases dramatically with increasing molecular size of the sample, if current electron microscopic techniques are used for its visualization. Early attempts in this direction led to network-like images (Bram & Ris, 1971). We shall outline here a new procedure allowing, in principle, the isolation and observation of entire, undistorted chromosomes from resting (G0) cells. It is beyond dispute that the use (Finch & Klug, 1976; Thoma et al. 1979) of fragments resulting from extensive nuclease digestion has represented a significant advance. However, is this method adequate for inferring the geometry of the higher-order structure of native chromatin? In the first place, for short polymer chains that can assume different conformations, the stable state is expected to be a function of the molecular weight. Further, eukaryotic DNA is organized into discrete, close structures (loops) that impose topological constraints on the chromatin strands, dictating the final conformation. By opening the loop the constraints are removed, and the conformation must necessarily change, unless one postulates that the genome of resting cells is always relaxed – a very arbitrary assumption.

In this work we used calf thymus as the material. There are both practical and heuristic reasons for this choice. Its chemical composition is well established (Bonner et al. 1968); detailed protocols reporting non-degradative extraction procedures as well as related analytical methods (Bonner, 1979) are available. Moreover, calf thymus cells are not cycling, so that chromatin from this source represents a quite good model for investigating the structural features eliciting condensation, which is thought to be the device by which gene expression is suppressed (Bonner, 1979).

There is no a priori impossibility in isolating single chromosomal strands; the three-dimensional reconstruction of the architecture of nuclei from Triturus cristatus erythrocytes, for instance, revealed the presence of distinct chromatin bodies, corresponding to the set of condensed interphase chromosomes, attached to discrete sites of the nuclear envelope (Murray & Davies, 1979); this observation seems to be, in general, valid and has recently been substantiated by computer image analysis of Feulgen-stained HeLa cells (Nicolini, 1980). Hence, experiments can be envisaged
in which single chromosomes are released from the nuclei in an untangled form. But in the course of the extraction one meets with several pitfalls, and we wish to indicate them and a possible way of avoiding them.

At variance with previous work, we avoided preparative conditions that were too drastic, and resulted in the loss of proteic components from the polynucleosomal complex. Non-ionic detergents and very low ionic strengths, in the presence of EDTA, are required in order to obtain regularly swollen nuclei. Under these conditions the phospholipid components of the nuclear envelope are removed; but a residual proteinaceous frame is still firmly associated with the bulk of chromatin. At this stage the nucleus is comparable with a rubber-like network under stress. If the cross-linking frame is broken too abruptly the elastic recovery of the strands will cause entanglements and extensive, spurious fibrillation. As a consequence, the application of both mechanical and shearing forces should be avoided as far as possible, and the nuclei harvested at very low rotor speeds.

A second point to which little attention has been given in current preparative methods is the mode of diluting the nuclear pellet down to the desired concentration. Direct, slow addition of the solvent to the pellet is a poor procedure. Chromatin fibres will start to spill out of nuclei at a still high concentration; entanglements and stretching of the whole system under the effect of the osmotic forces are unavoidable (the artifact is revealed in the light microscope by the presence of thick, birefringent fibres). On the contrary, native chromatin solutions are obtained if the nuclear suspension is dispensed dropwise into a large volume of the final buffer. This ensures both the isotropic and independent swelling of the chromatin bodies. In addition, a very mild digestion of the nuclei with micrococcal nuclease has been successfully introduced to promote the detachment of the strands.

A carbon surface is a hydrophobic substrate, and the high value of the contact angle for aqueous solutions gives rise to tremendous shearing forces when the solvent is removed prior to staining or shadowing the specimen. The use of carbon films submitted to a glow-discharge in order to increase their wettability does not greatly improve the results; elongation and distortion of the sample are still evident (Worcel et al. 1981). The same comment holds for a recently introduced technique, employing benzylidimethylalkylammonium chloride in association with a carbon support (Vollenweider, Sogo & Koller, 1975). A phospholipid monolayer can be formed easily at an water–air interface; it is thermodynamically stable and can be firmly transferred to a carbon-coated grid. Recently, we envisaged a new method involving a lecithin monolayer as the substrate (Cavazza et al. 1979). The chromatin images acquired in this way were found to be absent from distortion and artifacts. We now present in detail the structural information gained from a systematic investigation of chromosomal fragments observed in different conformational states.

**MATERIALS AND METHODS**

**Preparation of nuclei and native chromatin**

Fresh calf thymus was obtained from the local slaughterhouse. Fats, contaminating tissues and blood vessels were carefully and quickly removed so that thymus could be frozen in liquid nitrogen.
not later than 30 min after the animal was killed. The tissue was stored at —30°C and used within a few hours.

Native, unsheared chromatin was prepared from calf thymus nuclei isolated by a modification of the method described by Panyim, Bilek & Chalkley (1971). All manipulations were performed at 4°C. About 15 g of frozen tissues were cut in small pieces and homogenized in a Waring blender with 300 ml of grinding medium (0.25 M sucrose, 5 mM MgCl₂, 10 mM-Tris-Cl (pH 8), 5 mM- NaHSO₃). The suspension was then filtered through eight layers of sterile gauze and centrifuged at 430 g for 10 min. The nuclear pellet was washed with 150 ml of washing medium (0.25 M sucrose, 5 mM MgCl₂, 10 mM-Tris-Cl (pH 8), 5 mM-NaHSO₃, 0.3% Triton X-100) and centrifuged at 430 g for 10 min. The washing process was repeated once more. The purification through 1.7 M sucrose was omitted to avoid any possible shearing effect on the nuclear chromatin. On the other hand, it has been demonstrated by Slayter, Shih, Adler & Fasman (1972) that the standard DNA: protein ratio (100:147) (Bonne et al. 1968) is unaffected by omitting the sucrose-pelleting step. The nuclear pellet was resuspended in 40 ml of a buffer composed of 10 mM-Tris-HCl (pH 8), 0.25 M sucrose, 5 mM-NaHSO₃, and centrifuged at 430 g for 5 min. The process was repeated three more times. Nuclei were then lysed by gentle suspension in a volume of 1 mM-Tris-Cl (pH 8), large enough to obtain the required dilution for electron microscopy (about 4 μg/ml DNA).

In some cases Li₂SO₄ (final concentration 3 mM) was added to the lysis buffer. In the relaxation experiments 1.36 × 10⁻⁷ M ethidium bromide (SIGMA) was present in the medium, the concentration of DNA being equal to 4 μg/ml. Selective removal of H1 histone was performed in the presence of either 0.6 M-NaCl or 0.25 M-Li₂SO₄.

**Nuclease digestion**

Nuclei obtained from the washing step (as described above) were resuspended in grinding medium and pelleted at 430 g for 5 min; the process was repeated once more in order to eliminate Triton X-100. The pellet was then washed twice in a buffer composed of 0.25 M sucrose, 1 mM-Tris-Cl (pH 8), 1 mM-CaCl₂. Nuclease digestion was essentially performed as described by Noll, Thomas & Kornberg (1975), at a DNA concentration of 1.2 mg/ml. Nuclei were suspended in 0.25 M sucrose, 1 mM-Tris-Cl (pH 7), 1 mM-CaCl₂ and after a preincubation at 37°C for 5 min, 5 units of micrococcal nuclease (SIGMA) per ml of nuclear suspension were added. After 20 s the digestion was stopped by adding EDTA (final concentration 2 mM), pH 7, and chilling on ice. Nuclei were then pelleted at 1000 g for 5 min and lysed in 1 mM-Tris-Cl (pH 7), 0.2 mM-EDTA (DNA concentration about 4 μg/ml). Reference chromatin samples for electron microscopy were prepared from undigested nuclei in 0.25 M sucrose, 1 mM-Tris-Cl (pH 8), 1 mM-CaCl₂ by gentle lysis in 1 mM-Tris-Cl (pH 8), 0.2 mM-EDTA.

**Electron microscopy**

The phospholipid monolayer (Cavazza et al. 1979) was prepared as follows. Twice-distilled water was poured into a Petri dish and the surface was repeatedly cleaned by pushing a paraffinized glass bar over it. L-α-lecithin, C₄₀H₇₂NO₂P (Fluka AG) was spread on the surface from a 5 × 10⁻⁴ M solution in purified chloroform, the initial area per molecule being approximately 12 nm². The monolayer was formed by compressing the surface solution up to a value of 4–3.5 nm² per molecule and picked up on carbon-coated nickel grids, held by a small magnetic bar. A droplet of chromatin solution was applied to the grid by a large bore pipette and allowed to adsorb on the monolayer for about 15 min. The excess solution was gently removed by filter paper, the grid washed with 2–3 drops of water and air-dried. The grids were shadowed with platinum at an angle of 16° in an Edwards 306 vacuum coater, from two directions 90° apart. This method greatly enhances the visualization of fine details compared with conventional rotary shadowing. A Siemens 102 electron microscope, with 20 μm objective aperture, operating at 80 kV was used.

**RESULTS**

**Observing large regions of the chromosome from resting cells: a regular folding pattern underlies the higher-order structure**

If unsheared chromatin is properly diluted with low-salt media (see Materials and
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Methods) and the solution (4 μg/ml DNA) gently shaken at 4°C in large-volume flasks, marked time effects on the morphology can be seen. At zero time most of the material is found in a compact form; after 3–4 h a large population of distinct fibres is observed. A second, slower process, taking about 3 days to go to completion, leads to the disappearance of higher-order structures. At this time the chromosomal fragments are detectable as isolated bundles of closely apposed 11 nm filaments, showing local orientation on the grid (Fig. 1ε). This behaviour is well monitored by a recently introduced viscosimetric method (Parodi et al. 1981). The intrinsic viscosity of suspensions of rat liver nuclei at low ionic strength increases from 90 to 270 dl/g within 4 h, approaching a stable value (400 dl/g) in 3–4 days (S. Parodi, unpublished results). What is revealed by these experiments is the decondensation of the chromatin bodies, leading to long fibres, and the subsequent unwinding of these latter. The addition of small (about 3 × 10⁻³ mol/l) amounts of Li₂SO₄ to the buffer solution markedly enhances the second process. This effect, unpredictable by current theories on the solutions of polyelectrolytes, is well explained in terms of binding of the sulphate anion to the charged amino groups of the histone proteins, as already demonstrated for model systems in our laboratory (Conio, Patrone, Rialdi & Ciferri, 1974a; Conio et al. 1974b).

Careful measurements of thickness revealed a situation more complex than expected. In addition to the 30 nm strand (termed here type IV) we observed chromatin strands having diameters roughly centred around 24, 18 and 11 nm (type III, II and I, respectively). Provided that the length of the sample is sufficiently small (below a few μm) the fibres are very frequently interlocked, giving rise to complex unwinding figures; the structural implications of this finding will be discussed below.

We observed giant chromosomal fragments (contour length from 18 to 60 μm). The type II fibre shown in Fig. 1A is about 52 μm long; it appears broken at the arrowed points, originally connected through a short region that has been lost under the electron beam. Now, the DNA content of calf thymus nuclei is close to 6·5 × 10⁻¹² g. The diploid chromosome number of Bos taurus is 60. By taking into account, provisionally, the suggested contraction ratio of DNA in going from its naked state (B form) to the first (interphase) level of condensation of the polynucleosomal strand (Bak, Zeuthen & Crick, 1977), we find that the mean contour length of a chromosome is, in this case, 9 × 10⁻² cm. What we are observing, therefore, in Fig. 1A is a fragment corresponding to about one twentieth of the mean chromosomal size.

Several morphological features of the fibre are already visible on inspection of the drawing. At locus a a short fragment splits into two 18 nm strands. Their path is a non-random sequence of arc-shaped segments, defined by inflection points, resulting in a characteristic rippled appearance. In a few cases the segments are almost straight, but the demarcation points are still visible as short, asymmetrical elements protruding from the fibre. Fig. 1b shows this organization in detail. The length distribution of the segments is quite narrow; 22 determinations yielded a mean value of 1150 nm; the range is from 1100 to 1330 nm, and it is very probably overestimated, because of the incidence of parallax errors, unavoidable when distant regions of the fibre are considered. It is remarkable that this ripple-like morphology is observed independently
of nuclease digestion; the fibre shown in Fig. 1c has been obtained by direct swelling of undigested nuclei (reference chromatin; see Materials and Methods). At the loci marked \((a-d)\) a type I fibre folds back, almost doubling its thickness between the letters and the arrowed points. From the morphological standpoint, it is worth noting the recurrence of both: (1) a bent organization of the strand and (2) the values of the contour lengths between the inflection points; \(ab, bc\) and \(cd\) are equal to 1150, 1150 and 1200 nm, respectively, falling in the range determined for nuclease-digested chromatin. The same comment applies to the type I arc-shaped segment shown in Fig. 1d, \(a'b'\) being equal to 1300 nm. In fact, large regions of the genome, after swelling in low-salt media, are observed as long trains of segments of almost equal size. In other cases the demarcation points correspond to fragments of a 15 nm thick lamina (Fig. 2), a regular repeat along the strand being still present; the distances between laminar pieces (marked by capital letters) are roughly multiples of 550 nm. The high-resolution micrographs shown in Fig. 2c, d and e confirm that we are not dealing with an artifact. The bordering (type IV) fibre and the fragments interpenetrate; entry and exit points are well-defined.

Interestingly, rat liver chromatin does not escape the rule that a long-range regular pattern is clearly discernible along the fibres (see accompanying paper).

The multistranded structure of native chromatin fibres; evidence for a coiled-coil conformation

Spontaneous unfolding without and with HI removal. It is obvious that ordered states may be assumed by an undistorted polymer chain as well as by a chain folded on itself. Both short- and long-range interactions are effective in the formation of stable helices. Consideration of this simple point, however, leads to the first testable prediction.

Short-range interactions are the main source of stabilization of the polynucleosomal flat helix, so it is quite reasonable to expect that its geometry changes in a continuous fashion as a function of the ionic strength, from the unordered 11 nm nucleofilament to helices having an increasing number of nucleosomes per turn (Thomaef al. 1979). The micrographs shown in Fig. 3 do not bear out this prediction. What is invariably observed at low ionic strength is neither a continuous change of the strand thickness with time nor, assuming very fast transition kinetics, a constant low value (11 nm), but rather an unwinding process involving the subdivision of a main fibre into a discrete number of subfibres. Fig. 3a contains a lot of important details: between \(b\) and \(c\) the structure is coiled; the main helical groove is visible at the arrowed points.

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Fig. 1. A. Scale drawing of a giant type II chromatid fragment; the micrographs in B correspond to the hatched region of the drawing. Bar, 1 μm. Nuclease-digested chromatin diluted in 1 mM-Tris-HCl (pH 7), 0.2 mM-EDTA, 3 mM-Li2SO4. Bar, 5 μm. C. Reference undigested chromatin (see Materials and Methods) diluted in 1 mM-Tris-HCl (pH 8), 0.2 mM-EDTA; note the sharply kinked morphology of the type I fibre. D. Native chromatin (type I fibre) in 1 mM-Tris-HCl (pH 8), 3 mM-Li2SO4. E. A polynucleosomal field observed after prolonged exposure (3 days) to the swelling buffer (1 mM-Tris-HCl (pH 8), 3 mM-Li2SO4). Bars (c, d, e), 200 nm.
Between a and b the type III fibre unwinds; the drawing (Fig. 3b) shows that the loops may be resolved into three single 11 nm filaments having approximately equal contour lengths (870, 880 and 900 nm for 1, 2 and 3, respectively). The straight regions ab and bc are of equal size (about 600 nm). This suggests that co-operative unwinding cannot propagate without limit along the whole length of the strand; rather the process occurs within defined regions, which behave as independent units.

The detail shown in Fig. 3c displays the unwinding of a type IV fibre. The path of the four subfibres is presented in the drawing (Fig. 3d). At the point marked a the strands separate; subfibre 1 is almost straight, at b' a second subfibre leaves the short three-filament region and reassociates with subfibre 1 at b. The same trend holds for the fibres 3 and 4. Finally, Fig. 3e shows that the type II fibre is a single, 11 nm filament folded on itself; note the small terminal 'eye'. So a class of coiled-coil structures is generated by winding in two, three and four regions belonging to the same strand. This concept is at the basis of our model, which we term 'rope-like'.

Consideration of further, striking regularities is appropriate. When the unwinding process is induced by electrostatic repulsion at low ionic strength, without any change in the composition of the DNA–histone complex, the observed loops are asymmetrical. Whenever a fibre splits into two or more subfibres the branch points are connected through an almost straight segment subtending an arc-shaped region. This is evident in Fig. 3a, b. At first glance this finding is puzzling. Why should the unwinding of a helical structure result in a loss of symmetry? The answer is found by measuring the arc-to-chord length ratio on several loops. The mean value of 40 determinations is 1.6 (range from 1.5 to 1.75). Obviously, this constancy is comforting, and from the value itself a possible conformational interpretation can be devised, and will be discussed below.

Removal of H1 histone invariably leads to symmetrical unwinding figures. An example is given in Fig. 4a; in this case the final medium contained 0.6 M-NaCl. The type IV fibre splits into two type II fibres, the contour lengths of the parent strands

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Fig. 2. A. Scale drawing of a type IV fibre showing the attachment of 15 nm laminar fragments placed at regular intervals. This structure is detailed in the micrographs (b, c, d, e). Nuclease-digested chromatin diluted in 1 mm-Tris-HCl (pH 7), 0.2 mm-EDTA. Bar, 1 µm. F. Reconstruction of the same laminar fragments shown in A; entry and exit points of the strand are marked by open and filled circles, respectively. Bars: b, 0.5 µm; c, d, e, f, 100 nm.

Fig. 3. A. Spontaneous unwinding of a type III fibre. The corresponding drawing (b) shows the subdivision of the strand into three mononucleosomal filaments having equal contour lengths. c. The unwinding of a type IV fibre. The path of the subfibres is shown in the drawing (d). e. A type II fibre resulting from the winding up of a type I fibre on itself. a–e refer to native chromatin in 1 mm-Tris-HCl (pH 8), 3 mm-Li2SO4. Bars: a, c, e, 100 nm.

Fig. 4. A. The effect of H1 histone removal on the unwinding of a type IV fibre; note that the contour lengths of the 18 nm parent strands are equal. H1 depletion carried out in 1 mm-Tris-HCl (pH 8), 0.6 M-NaCl. Bar, 200 nm. B. H1-depleted type III fibre; in this case the final buffer contained 1 mm-Tris-HCl (pH 8), 0.25 M-Li2SO4. Bar, 200 nm. C. The effect of limited nuclease digestion on a type IV fibre. Note the partial unwinding process leaving a notch along the main strand. Bar, 100 nm.
Fig. 3. For legend see p. 89.
Fig. 4. For legend see p. 89.
being the same within each loop. Comparable results are obtained in the presence of 0.25 M Li2SO4, a more selective agent for H1 depletion. Although the specimen shown in Fig. 4A is coated heavily with platinum, it is nevertheless worth studying; a short-range regular unwinding of a type III fibre is apparent, especially if the micrograph is viewed at glancing angles in a direction parallel to the fibre axis.

Limited chromatin digestion as a structural probe. Additional evidence favouring the coiled-coil model was obtained by mild degradation with nuclease, the optimal experimental conditions being the same as those used in the preparative step (see Materials and Methods). This probe is particularly valuable for large chromatin fragments. Spontaneous unwinding depends on the rotation of two domains of the strand with respect to each other, a motion determined, to a large extent, by the size of the fibre. Hence, above some upper size-limit, no appreciable conformational change is expected to occur at short times (Figs 1, 2); a prolonged exposure to solvent, on the other hand, increasing the incidence of endogenous degradation. It is important to notice that, in order to optimize the information amount of the experiments, the linear density of the double-strand cuts must be kept low (roughly, 1 break per 10 μm of type IV fibre, or less). This level is established both by the tendency of the DNA breaks towards clustering within limited regions of the fibre, and by the effective target size. The structural determinants of this important quantity, some orders of magnitude smaller than the chromatid length, will now be considered.

Relevant results obtained by the limited-digestion method are shown in Figs 4c and 5. Each double-strand cut is marked by an arrow; arabic numerals indicate the number of residual strands. The drawing in Fig. 5B shows the path of the subfibres; by measurements of thickness we again find the discrete values corresponding to the four types of fibres described above. All the micrographs refer to the same specimen, 8 μm long, having a uniform thickness of 30 nm.

The situation shown in Fig. 4C is very straightforward. At the arrowed points two nearby breaks have been introduced by the enzyme. A partial unwinding process reveals two closely associated subfibres, together with the notch on the main strand. This micrograph alone could rule out the identification of the 30 nm fibre as a single-stranded helix. It is clear that, in this case, one break should be enough to cut the strand in half. This does not happen because the molecular arrangement corresponds to a four-strand coiled-coil.

From Fig. 5A we gain a deeper insight into the mode of organization of the fibre. The drawing (Fig. 5B) is self-explanatory. Degradation has caused the loss of long segments of the 11 nm subelements. A few morphological features should be noted. At the points marked a and a', two residual mononucleosomal strands fold back on themselves (unbroken and broken lines). Their contour lengths (bad and ba'c) are equal to 1250 nm, the value frequently observed for native fibres. Irrespective of this numerical coincidence, by rejoining the end-points of the fibre, c and d, we attain a

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Fig. 5. A. A type IV fibre digested by nuclease. Details of the fibre organization are shown. The drawing (a) demonstrates the digestion pattern; further basic morphological elements, the apexes (a, a'), are evident.
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Fig. 5

Fig. 5A

Fig. 5B
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qualitative interpretation of the inflection points of the fibre shown in Fig. 1A, B; at high resolution they are now visualized as regions from which a single filament leaves the helical path giving rise to short hairpin-like elements (apexes).

Because the number of chain breaks introduced by our procedure is small with respect to the target size, we rule out, on principle, the incidence of artifacts induced by chain degradation. In some cases, however, single polynucleosomal fibres undergo limited structural rearrangements. In Fig. 1c, d, reassociation and/or coiling, hinged at a, b, c, d and a', b', is evident. This is expected. The fragments are short and their motion unrestricted. Whether these situations correspond to true equilibrium, or rather to a compromise between equilibrium and kinetics, cannot be decided on the basis of the present data.

DISCUSSION

It has been the objective of much work in recent years to understand the spatial organization of chromatin within resting nuclei. It now appears well-established that the interphase genome has a complex nuclear frame, composed of nuclear pores associated with a proteinaceous lamina, fibrillar nucleoli and an intranuclear matrix (Comings & Okada, 1976). Furthermore, independent experiments have revealed that the chromatin strands are structured into closed subelements (loops) introducing additional topological constraints. Comings & Okada (1976) described 'rosettes' (i.e. DNA loops tightly bound to a dense nuclear matrix) in mouse liver nuclei solubilized in 2M-NaCl. Benyajati & Worcel (1976) were able to isolate the intact interphase genome of Drosophila under milder conditions and demonstrate its subdivision into independent loops by relaxation experiments in the presence of ethidium bromide or DNase I. The reliability of this kind of observation has been disputed. The objection that improper preparative methods could lead to artifactual protein segregation as well as to a general rearrangement of the organization of the genome has been put forward. Recent analytical work, however, has to a large extent clarified this point. Razin et al. (1981) were able to isolate a class of proteins directly associated with skeleton-attached DNA; the complexes are resistant to very drastic extraction procedures; the authors suggest further that some of these proteins could be responsible for specific DNA–skeleton interactions. These findings, establishing a clear distinction between DNA-bound proteins and the nuclear frame, argue against theories interpreting the loop organization as an artifact.

It is evident that the branched structures shown in Figs 3 and 4 give rise, after relaxation, to clusters of stretches of closed-circular DNA (Benyajati & Worcel, 1976). In fact, as we have already noted, spontaneous unwinding occurs only within defined domains of the fibre; this requires macromolecules of different nature to give rise to tie-points by firmly crosslinking the folded configuration. The branch points are often associated with laminar fragments. Our observations, however, argue against a direct involvement of the nuclear envelope in the fastening of the linear strand. Actually, branching of the fibres (within defined domains) occurs in the absence of residual laminar pieces as well (Fig. 3A, c). The exact nature of the
intrastrand crosslinking macromolecules is at present unknown; they could belong to the family of proteins tightly bound to DNA, described by Razin et al. (1981). The present observations do not offer a sufficient experimental basis for an overall description of the path of the chromatin strands linked to distinct components of the frame. In addition to laminar structures, the presence of fibrillar elements should be noted. Fig. 5A shows such an element associated with a 30 nm chromatin strand; a connection point is marked by an asterisk, but, in fact, protein fibres are seldom detectable. Our extraction procedure, of course, was not designed to maintain their delicate structure. Nevertheless, our results suggest a minor role for the intranuclear matrix in the spatial organization of chromatin, at least for resting cells.

In several cases, when well-defined laminar fragments can be visualized (Fig. 2), it becomes possible to attempt a reconstruction to restore the situation existing prior to the disruption of the nuclear envelope. After photographic enlargement of the major fragments and by patient recombination of their profiles we obtained the results shown in Fig. 2r. We present this figure with reservation; some edges may be slightly altered by chipping and furthermore three fragments were too small to allow an unambiguous determination of their shape and could not be taken into account. By connecting the entry and exit points (filled and open circles, respectively) with the continuous chromatin strand, according to the succession shown in Fig. 2, one achieves a 'drapery-like' (Nicolini, 1979) organization, i.e. a system of contiguous loops anchored to a surface. The mean contour length of the loops is 1420 nm, a value that, although probably overestimated, is comparable to the period determined for the fibre shown in Fig. 1A. This reconstruction exemplifies a method for interpreting a very frequently observed situation. Swollen, unsheared chromatin from rat liver nuclei is associated distinctly with laminar fragments and the investigation of these structures is in progress by more sophisticated methods of image-processing (B. Cavazza, C. Nicolini, E. Patrone & V. Trefiletti, unpublished results). The somewhat naive 'game' shown in Fig. 2r can be extended. The reconstitution gives rise to a cluster of untangled loops. Further, both the entry and exit points are grouped into domains, so that many segments belonging to different loops project from the surface at very close points; this is in line with the results of Olins & Olins (1979) and Lepault et al. (1980); the former authors were able to visualize, by stereo electron microscopy of thin sections of chicken erythrocyte nuclei, closely packed parallel fibres anchored to the nuclear envelope.

We cannot measure the relative amounts of chromatin bound to the nuclear envelope and to the fibrillar frame. But a simple calculation based on both the mean value of the contour length of the interphase genome and the surface density of the attachment points (Fig. 2r), leads to the conclusion that the bulk of chromatin may be accommodated without competing with the envelope for space. Further, the mean segment size is about 1 μm, so that, by assuming major involvement of the envelope, we expect the more densely populated nuclear region to correspond to a shell 0.5 μm thick. Recent investigations of the radial density distribution of chromatin from resting cells or interphase nuclei indicate that this is indeed the case (Kendall et al. 1980).
None of the results reported has given any insight into the architecture of the folded genome; we have limited ourselves to describing a hierarchy of structures of increasing complexity. The simplest case corresponds to a polynucleosomal chain folded on itself, clamped at a single site by specific DNA–protein interactions; but the situation reported in Fig. 1A is much more intriguing, because the folding process involves an extremely long sequence of distinct segments. Complementary relaxation experiments in the presence of ethidium bromide proved to be particularly useful in revealing the path of the strand between successive tie-points, allowing a preliminary model for the folding of the giant chromosomal fragments to be put forward.

The term self-assembly, although unusual in this field, is quite appropriate if applied to the set-up of the DNA–protein complex. The histone octamer can be reconstituted in vitro in a form indistinguishable from the native one; its geometry directs in a precise fashion the folding pattern of the DNA duplex. We propose that the higher-order structures of chromatin, which are, puzzlingly, both folded and coiled, is dictated in turn by the self-assembly properties of the basic 11 nm polynucleosomal strand.

The structure of the genome in resting cells is the outcome of a sequence of coordinated biochemical events; we believe that from the chemical–physical standpoint the final structure can be built up, step by step, on the basis of the morphological elements identified in this work. In a first stage the mononucleosomal strand must fold. The long semi-flexible chain is subjected to stringent spatial constraints; therefore, in order to attain any (untangled) folded configuration on a finite time-scale it is required that the strand be segmented into a large number of 'freely joined' sub-elements – a device that greatly enhances local flexibility. Evidence for the presence of very sharp inflection points along the 11 nm nucleofilament has been fully documented (Fig. 1C, D). They could arise from A+T-rich linkers (Moreau et al. 1982) or from tightly bound DNA–protein complexes.

By making allowance for chain flexibility, several folding modes become possible, depending on the number of the segments involved, the presence of a regular repeat distance and the relative disposition of the joints. The drawing in Fig. 6A shows the genesis of a type II fibre; in this case the equal-sized elements are half-staggered, but this is very probably a particular case because nothing prevents them from overlapping or assuming intermediate positions. The structure sketched in Fig. 6C is obtained simply by coiling a folded strand; the final result is a continuous fibre interspaced by short loops, the apexes, to which reference has already been made. An apex will emerge in the corresponding joint whenever a folded strand winds up on itself.

Experimental evidence supporting our interpretation is compelling. The type II
Fig. 6

Structure of chromatin from resting cells

6A

6B

6C

6D

6E

Fig. 6
fibre shown in Fig. 6B was observed after partial relaxation in the presence of ethidium bromide, under conditions where approximately 30% of the strong affinity sites of chromatin are saturated (Lawrence & Daune, 1976). The fibre unwinds between the arrowed points; two joints and one apex are evident, a, b and c, respectively; the single filaments have equal contour lengths (2000 nm). Upon recoiling as above two apexes are restored at a and b; the interdistances ab and bc are in the ratio 1:2, an additional indication that often the lengths of adjacent segments are either equal to or a multiple of a fixed value. Our model accounts for the existence of stable loops, and further allows a testable correlation to be proposed between the loop size, the effective target size and the length over which the strands undergo unwinding. Fig. 6D, E shows the configuration of 'naked' apexes, frequently obscured by residual envelope fragments; when they come off the strand a supercoiled conformation at the joints is observed. Clearly, the structure of the chromatin–skeleton complex needs further investigation; the example, shown in Fig. 2B, does not allow us to recognize the path of the strand within the laminar domains, nor does Fig. 5 reveal in detail the anchorage to a fibrillar element.

The zero-order model shown in Fig. 7 was suggested, in the first place, by considering the asymmetrical unwinding of coiled-coil fibres; moreover, in spite of the lack of a detailed conformational analysis, it is compatible with several experimental results (see accompanying paper) and with simple thermodynamic considerations. Although it differs from the twisted ribbon structure proposed by Worcel et al. (1981), we have followed the suggestion of these authors; namely, that the driving force for any given helical array of linked nucleosomes arises from interaction among their complementary flat surfaces. The model depicts a type III fibre; the helix has seven nucleosomes per turn and a diameter of 23 nm; the internucleosomal distances are not in scale, for the sake of clarity. Neither the path of DNA running along the nucleosomal strands nor the location of H1 are shown. We did this because the folding pattern of DNA is an additional topological determinant of the structure. The change of L (linking number) for a close relaxed DNA duplex after assembly with histones approaches \(-n\), where \(n\) is the number of nucleosomes, and this relationship must be used as a selection rule for different helical states (Worcel et al. 1981). The determination (Crick, 1976) of the \(\Delta L\) values inherent in our structures is in progress.

The constancy of the arc-to-chord ratio is easily interpreted as follows. The nucleosomes can be approximated to flat cylinders of dimensions 5.7 nm \(\times\) 11 nm \(\times\) 11 nm (Finch et al. 1977), and in the helical conformation the balance between attractive potential and steric hindrance leads to a mean interdistance of \(\sim 1\) nm for nucleosomes belonging to the same strand (E. Patrone, B. Cavazza & V. Trefiletti, unpublished results). Whenever a similar coiled structure unwinds one or more subfibres will lose the helical conformation; the nucleosomes sliding onto each other will change their orientation progressively with respect to the fibre axis; they are finally viewed as strings of 11 nm edge-to-edge elements. Minor rearrangements of the residual filament allow them to retain the stacked conformation; this explains the apparent asymmetry of the process. More quantitatively, by adding to the nucleosomal height the internucleosomal distance we obtain 6.7 nm. The ratio of 11 to 6.7 is equal to 1.64,
Fig. 7. Zero-order model of a type III fibre; the internucleosomal distance is not in scale. The model demonstrates the asymmetrical unwinding process observed for native chromatin at low ionic strength (see Fig. 3). Note the stacked configuration of the nucleosomes belonging to the straight region. DNA path not shown.
in quite good agreement with the experimental value. This result may be verified in a formally independent way by considering that the number of nucleosomes must be the same for all the subfibres within a given loop. By taking into account the above ratio we obtain from Fig. 3A, 67, 65, 68 and 62 nucleosomes for subfibres 1, 2, 3 and 4, respectively. This range is narrow and it is very probably determined by end-effects, i.e. it depends on the intermediate orientation of the nucleosomes near the branch points, which becomes negligible for large loops. The symmetrical unwinding process observed for H1-depleted chromatin confirms the structural role of this protein; it is possible that, as a consequence of its removal, the coiled state undergoes a relaxation process, the internucleosomal interaction favouring everywhere an orientation perpendicular to the fibre axis, so that the apparent asymmetry of the loops disappears. This interpretation requires further, more quantitative work.

In this paper an effort has been made to develop a general picture, which may relate to several outstanding problems in eukaryotic replication research. Within $S$ phase, clusters of contiguous units of replication (replicons) initiate DNA synthesis at the same time (Huberman & Riggs, 1968); furthermore, synthesis within a cluster is inactivated by single-hit kinetics (Painter & Young, 1976); evidence for a fixed site of DNA replication anchored to the nuclear matrix has been recently reported by Pardoll, Vogelstein & Coffey (1980). In the light of these points, a few questions may be raised. We may ask, for instance, whether the long trains of equally sized segments are the structural counterpart of a cluster. The target size of this latter ranges from 100 to 700 $\mu$m of DNA (Povirk, 1977), the number of replicons per cluster being between 10 and 20. Hence the DNA length per replicon falls in the range 5–70 $\mu$m. The segment length of the type II fibre shown in Fig. 1A is approximately 1 $\mu$m, leading to a value of about 20 $\mu$m of DNA, according to the contraction ratio (Bak et al. 1977). Clearly this is an acceptable result, although caution should be exercised when the properties of chromatins from different sources are compared.

Nothing is known about the mechanism of synchronized initiation within the cluster, nor does single-hit kinetics of inactivation lend itself easily to a structural interpretation. The necessity of a signal-generating central engine has been stressed (Wilkins, 1981), but its identification (Wilkins, 1981) with an axial element placed at the base of the loop (Adolph, Cheng & Laemmli, 1977) appears quite inconclusive.

The signal-amplifying properties of the coiled-coil model should not be overlooked. Assuming, for instance, that bidirectional DNA replication starts from the white apex in Fig. 6c conformational changes could be co-operatively induced into the black strand, resulting in a possible activation of further synthesis sites. Gene expression may be interpreted according to the model as well. Higher orders of coiling should underlie gene inactivation, while local disorder (for instance, the intercalation within a regularly spaced chromatin fibre of a 'transgressing' segment) could produce a protruding loop in the expressible form. Conceptually this corresponds to Bonner's (1979) interpretation of the function of interphase chromatin; our additional suggestion being that expression is modulated by the order–disorder balance within the system.
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