ASSEMBLY OF CHROMATIN FIBERS INTO METAPHASE CHROMOSOMES ANALYZED BY TRANSMISSION ELECTRON MICROSCOPY AND SCANNING ELECTRON MICROSCOPY

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ABSTRACT The higher-order assembly of the ~30 nm chromatin fibers into the characteristic morphology of HeLa mitotic chromosomes was investigated by electron microscopy. Transmission electron microscopy (TEM) of serial sections was applied to view the distribution of the DNA-histone-nonhistone fibers through the chromatid arms. Scanning electron microscopy (SEM) provided a complementary technique allowing the surface arrangement of the fibers to be observed. The approach with both procedures was to swell the chromosomes slightly, without extracting proteins, so that the densely-packed chromatin fibers were separated. The degree of expansion of the chromosomes was controlled by adjusting the concentration of divalent cations (Mg^{2+}). With TEM, individual fibers could be resolved by decreasing the Mg^{2+} concentration to 1.0–1.5 mM. The predominant mode of fiber organization was seen to be radial for both longitudinal and transverse sections. Using SEM, surface protuberances with an average diameter of 69 nm became visible after the Mg^{2+} concentration was reduced to 1.5 mM. The knobby surface appearance was a variable feature, because the average diameter decreased when the divalent cation concentration was further reduced. The surface projections appear to represent the peripheral tips of radial chromatin loops. These TEM and SEM observations support a "radial loop" model for the organization of the chromatin fibers in metaphase chromosomes.

INTRODUCTION

Chromosome Substructure: Nucleosomes and 30 nm Fibers

Nucleosomes are the fundamental structural units of the chromatin fibers that are folded into metaphase chromosomes (1–3). Nucleosomes are complexes of histones and DNA, each unit containing two molecules of histone H2A, H2B, H3, and H4, one molecule of histone H1, and ~200 base pairs of DNA. This repeating subunit of chromatin was initially identified by nuclease digestion experiments and electron microscopy. Digestion with micrococcal nuclease released monomer nucleosomes from both metaphase chromosomes and interphase nuclei. A "beads-on-a-string" appearance for the nucleosomal filaments was revealed by electron microscopy. X-ray diffraction and image reconstruction from electron micrographs demonstrated that nucleosomes are disk-shaped particles, 10 nm in diameter and 5.5 nm in height, with 1¾-to-2 superhelical turns of DNA wrapped around the outside of the core histone octamer.

A "solenoid" model for the higher-order structure of chromatin was proposed on the basis of EM observations (4). According to this model, the 30 nm fibers, which have been recognized for a number of years as the basic chromosomal fibers, are composed of a superhelix of the nucleosomal filament. The solenoid contains ~6 nucleosomes per turn. Hydrodynamic studies of chromatin fibers in solution (5–7) and additional EM investigations (8) provided convincing evidence for the superhelix or solenoid model. Light scattering results supported the proposal for a helical coiling of the nucleosomal filament (9, 10). X-ray diffraction showed the internal structure of nucleosomes and the packing of nucleosomes in chromatin fibers to be unchanged in the transition from interphase to metaphase (11, 12). In the intact nucleus, the 30 nm fibers are not only of structural significance, but, organized into higher-order domains, the fibers constitute functional units for DNA replication and gene transcription (13–15).

Models for Chromosome Higher-Order Organization

The formation of the solenoid from the interaction of the DNA molecule with histones compacts the DNA ~40-fold. But a total compaction approaching 10,000-fold is required to account for the amount of DNA in human metaphase chromosomes. One type of chromosome model considers that the helical coiling of the solenoid is continued to create a hierarchy of helices up to the final level of the assembled chromatid. Another general class of chromosome model views the chromatid morphology as resulting from direct folding of the 30 nm fiber into an

array of loops. These two models are illustrated in Fig. 1. Fig. 1 *A* shows the final level of coiling in a chromatid for the "helical coil" or "supersolenoid" model, while Fig. 1 *B* depicts the transversely oriented loops that would be found in a chromatid constructed according to the "radial loop" model.

The central aspect of the "supersolenoid" model is the proposal that chromosomes contain a tubular structure formed by the helical coiling of the solenoid. The presence of such structures was reported by Bak et al. (16) and by Sedat and Manuelidis (17) on the basis of light and electron microscopic studies of interphase nuclei and metaphase chromosome preparations. Bak et al. observed long, cylindrical structures in partially disintegrated chromosome preparations that had a uniform diameter of 400 nm. The coiled tube in Fig. 1 *A* illustrates the final level of twisting of the "supersolenoid" that would be required to create a chromatid. The model of Sedat and Manuelidis is similar, although the dimensions of the tube are different. Micrographs of interphase nuclei were interpreted as providing evidence for a 200 nm tube instead of a 400 nm "supersolenoid."

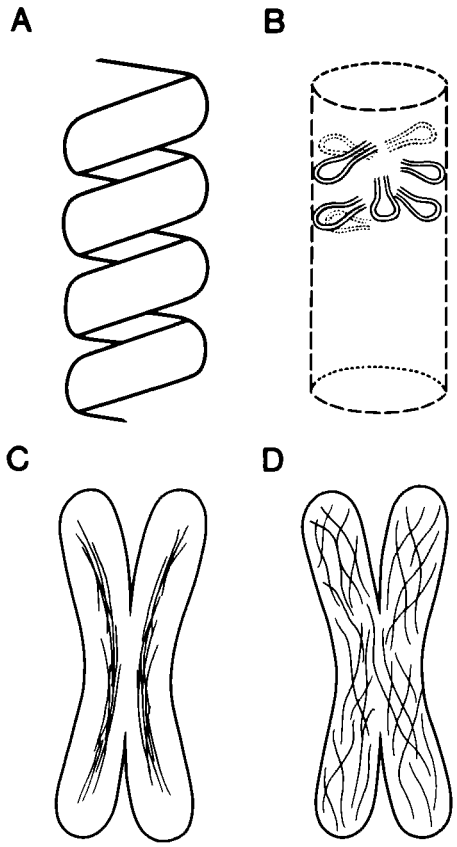

FIGURE 1 Diagram representing contrasting models for the higher-order arrangement of chromatin fibers in metaphase chromosomes and for the role of nonhistone proteins. (*A*) The "supersolenoid" model of chromosome structure. The final level of coiling of the 400 nm "supersolenoid" into a portion of a chromatid is depicted. (*B*) The "radial loop" model of chromosome structure. The chromatin fibers are directly folded into loops, the tips of which define the boundary of the chromatid. Two possibilities for the distribution of nonhistones which help to maintain chromosome structure are illustrated in panels *C* and *D*. The structural nonhistones in panel *C* are depicted as organized into a dense, central core. An alternative arrangement for the structural nonhistones is displayed in panel *D*. The proteins are distributed through the volume of the chromosome as a loose, fibrous network. In both models, the chromatin fibers would be constrained into the volume of the chromosome by interacting with the structural nonhistones. These proteins could, for example, cross-link the bases of loops.

No intermediate tubes are required to exist in the "radial loop" model. The 30 nm fiber is directly folded in a looped arrangement to form the characteristic metaphase chromosome morphology. The loops originate close to the central chromatid axis and extend to the boundary of the chromatid, which the peripheral tips of the loops thus define. The path of connecting the loops is unclear. The connections may produce a symmetric arrangement of loops, but no experimental evidence has been reported for an ordered packing of the looped fibers. This general type of model is similar to the earlier "folded fiber" model of DuPraw in proposing a direct folding of the basic chromosomal fibers. More recently, high-resolution scanning electron microscopy of individual chromosomes has revealed the presence of surface projections (18–20). These protuberances can only have arisen from the folding back of the underlying chromatin fiber.

The highly condensed nature of intact metaphase chromosomes has limited the information that can be obtained by electron microscopy. An extremely valuable approach was to extract, under mild solution conditions, the histones and other chromosomal proteins (21, 22). The residual particles still resembled the original chromosomes. EM of histone-depleted chromosomes showed the DNA to be arranged as loops that were at least 10–30 μm (30–90 kilobases) in length (22). The loops extended from a central "scaffold" region. Thin sections and scanning EM of intact chromosomes strengthened the evidence for a "radial loop" model (23).

Role of Nonhistone Proteins

What interactions are responsible for maintaining the characteristic shape of metaphase chromosomes? The models presented in the preceding section were concerned only with describing the pathway of the fibers. They were not concerned with the biochemical interactions that are required to assemble and stabilize the higher-order structure of chromosomes. Metaphase chromosomes contain, in addition to histones and DNA, the heterogeneous class of proteins known as nonhistones. Hundreds of distinct species can be recognized on two-dimensional polyacrylamide gels. The functions of most of these proteins are unknown.

Certain nonhistone proteins could have a structural role in maintaining metaphase chromosome morphology. Fig. 1 *C* and *D* show two general models for the structural involvement of these nonhistones. *C* shows the proteins to be organized into a dense, central core, while in *D* the

species constitute a loose, fibrous network that extends throughout the chromatid.

Studies of histone-depleted chromosomes and chromosome "scaffolds" have provided evidence for the existence of a network of structural nonhistones, as shown in Fig. 1 *D*. The central scaffolds of histone-depleted chromosomes retained the original morphology of the chromosomes, even though only a few residual protein species were present (24). The integrity of the particles did not depend upon RNA. Treatment with proteases, however, completely unravelled the histone-depleted chromosomes. Fluorescence microscopy of samples stained for protein revealed the striking resemblance of the scaffolds to the original chromosomes. Most interestingly, the scaffold structures did not depend upon the presence of DNA loops. Chromosomes were incubated with micrococcal nuclease or DNAase I before removing proteins, and the residual structures still resembled extracted chromosomes (24). The same subset of nonhistones was present as for histone-depleted chromosomes. Electron microscopy revealed fibrous particles with the shape of the original paired chromatids.

Additional experiments have been conducted to characterize the biochemical and structural characteristics of the metaphase scaffold. Using highly purified chromosomes, the scaffold was found to be composed primarily of two high-molecular-weight species. These proteins, Sc1 and Sc2, had molecular weights of 170,000 and 135,000 (25). The scaffolding structure was stabilized by metalloprotein interactions. Metal chelators or thiol reagents dissociated the particles, but the structure could be stabilized by Cu^{2+}. Microscopy revealed the retention of differentiated regions seemingly derived from kinetochores and the chromatid axis (26). Antikinetochore sera from patients with scleroderma were used to show that components of the kinetochore are found in the scaffold (27). The "core" in intact metaphase chromosomes observed by cytological silver staining appears to be equivalent to elements of the scaffold (28).

MATERIALS AND METHODS

Isolation of Metaphase Chromosomes

Minimum essential medium, supplemented with 5% fetal bovine serum, was used to propagate HeLa S3 cells in suspension culture. The medium, Joklik-modified for suspension culture, was obtained from the Grand Island Biological Company (Grand Island, NY), while the serum was purchased from Flow Laboratories (McLean, VA).

The scanning EM experiments required isolated metaphase chromosomes, and two methods were used to purify samples. Most experiments used chromosomes prepared with a buffer containing divalent cations (Mg^{2+}, Ca^{2+}) to maintain the particles in a condensed state during isolation. Cells were arrested in mitosis by adding colchicine (0.2 μg/ml) for 12–16 h. After cooling on ice, the cells were washed and resuspended in isolation buffer consisting of 50 mM NaCl, 5 mM Hepes (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), pH 7.4, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM PMSF (phenylmethylsulfonyl fluoride). Detergents, 0.5% NP40 (Non-Idet P-40) and 0.1% sodium deoxycholate, were added, and the hypotonically swollen cells were disrupted in a Dounce tissue homogenizer (Wheaton; Millville, NJ). A similar protocol was followed to isolate chromosomes by a second method which used a polyamine-containing buffer. This buffer was composed of 80 mM NaCl, 5 mM Hepes, pH 7.4, 0.5 mM spermine tetrahydrochloride, 0.15 mM spermidine trihydrochloride, 0.2 mM EGTA (ethyleneglycol-bis-(aminoethyl ether) N,N′-tetraacetic acid), 0.1 mM PMSF. Chromosomes were purified from the cell lysates by pelleting through 40% sucrose, after centrifugation to remove large debris.

Preparation of Specimens for Transmission EM

Thin sectioning experiments used hypotonically swollen mitotic cells, rather than isolated chromosomes, to avoid any distortions to the chromosomes that could occur during isolation. HeLa cells, arrested in mitosis, were treated with hypotonic buffer and nonionic detergent prior to fixation. Most experiments described in this paper used hypotonic buffer containing 1.5 mM Mg^{2+} though the concentration was varied from 5.0 mM to 0.15 mM in different experiments. The composition of the buffer was typically 10 mM NaCl, 10 mM sodium cacodylate, pH 7.0, 1.5 mM $MgCl_2$, 0.1 mM PMSF. Cells in this buffer were extracted with NP40 detergent (0.1%) for 30 min and then fixed with glutaraldehyde (0.8%) for 90 min. Treatment for another 90 min with osmium tetroxide (1%) followed washing the cells with 0.1 M sodium cacodylate, pH 7.0. The cells were immobilized in 2% agarose, and small blocks were prestained for 20 min with 1% uranyl acetate. After dehydration through steps of 20, 50, 75, and 100% acetone, the samples were cured in Epon 812 or Spurr medium for thin sectioning.

Preparation of Specimens for Scanning EM

Isolated chromosomes, obtained with the Mg/Ca procedure, were resuspended in buffer containing 5 mM Hepes, pH 7.4, and a $MgCl_2$ concentration of 1.5, 0.5, 0.15, or 0.05 mM. The particles were allowed to adhere to glass coverslides for 30 min at 4°C. Glutaraldehyde, 0.8% in the appropriate buffer, was then placed over the specimens for 60 min. After the coverslides were washed in buffer, osmium tetroxide (0.05%) was added for 30 min, followed by another wash in buffer. Critical-point drying in liquid CO_2 followed dehydration of the samples in ethanol and transfer into Freon 113.

TEM and SEM

For transmission EM, serial sections of embedded mitotic cells were obtained with a diamond knife. Carbon-coated grids were used to support the silver-grey sections (~60 nm thick). The grids were stained with uranyl acetate and lead citrate before being examined in the electron microscope.

Isolated chromosomes that had been critical-point dried were coated with gold-palladium in preparation for scanning EM. Equal amounts of 100% gold wire and 60%/40% gold-palladium wire were vaporized in a Denton apparatus. The thickness of the metal coating was determined from the diameters of latex spheres (109 nm original diameter) that were coated along with the chromosomes.

RESULTS

Transmission EM of Chromatin Fiber Organization

An overall view of a mitotic HeLa cell treated with hypotonic buffer containing 1.5 mM Mg^{2+} is shown in Fig. 2 *A*. This concentration of magnesium ion proved to be optimal for slightly separating the chromatin fibers. Under these conditions, the diameters of the chromatids had

increased ~30% above their native, compact state. NP40 detergent in the hypotonic buffer acted to solubilize much of the membranous material that surrounded the chromosomes. The monovalent salt (NaCl) concentration and pH could also affect chromosome morphology, but the dominant influence was the magnesium ion concentration.

The densely staining chromosomes within the mitotic cell of Fig. 2 *A* are seen to be sectioned both transversely and longitudinally to the chromatid axis. Most chromosomes, however, have a circular or oval shape. This would be expected because of the characteristic, elongated shape of HeLa chromatids, which are ~1 μm in diameter and

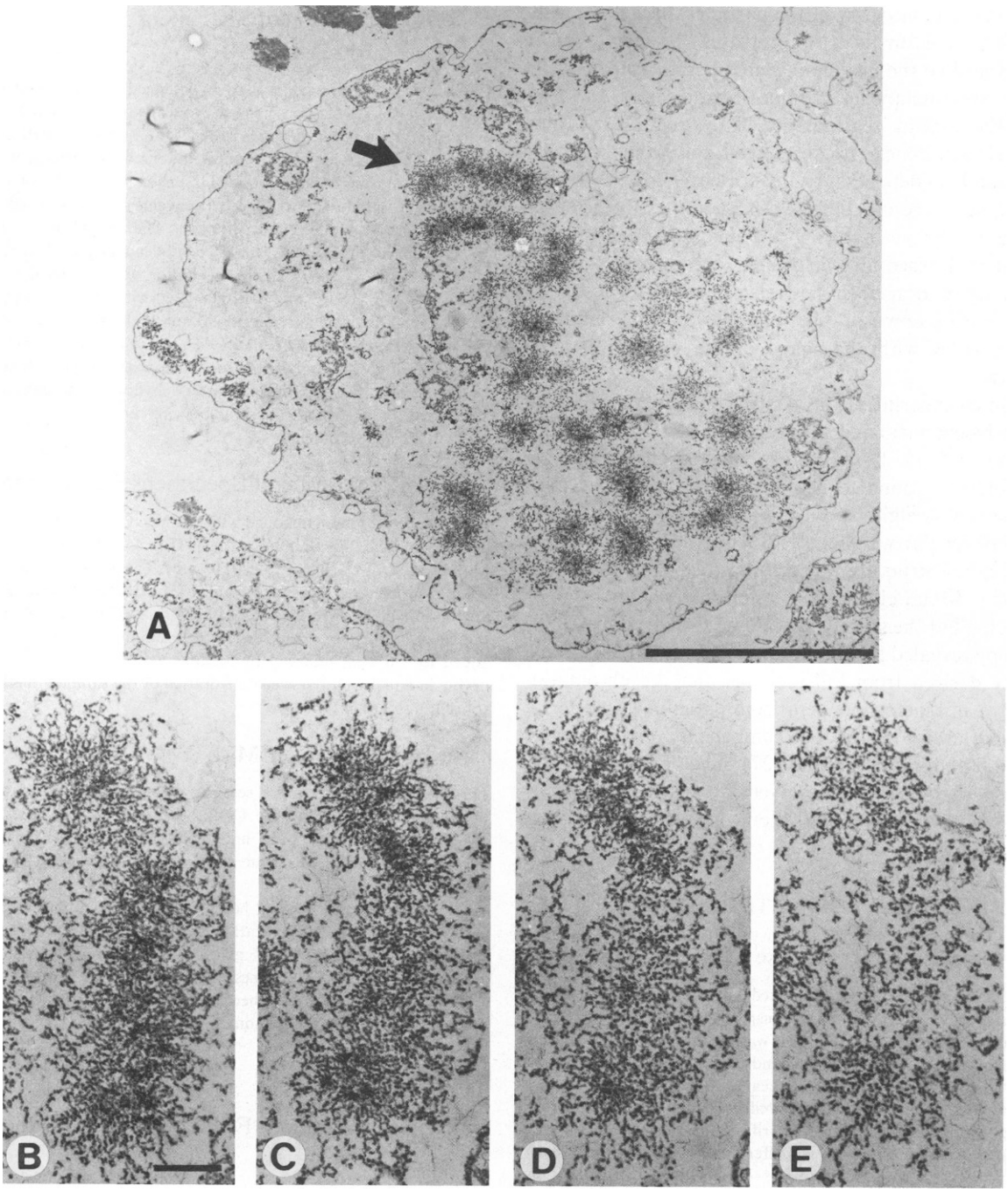

FIGURE 2 Serial sections through a mitotic HeLa cell chromosome that intersect the chromatid longitudinally. An overall view of the hypotonically swollen mitotic cell containing the chromosome is shown in panel *A*, and the chromatid shown enlarged in panels *B–E* is indicated with an arrow. The sample was prepared for transmission EM after resuspension in buffer containing 1.5 mM Mg^{2+}. The section in panel *B* is closest to the central chromatid axis, while panel *E* contains a section that grazes the periphery of the chromatid. The bar represents 5.0 μm in (*A*) and 0.5 μm in (*B–E*).

2–10 μm in length. A pair of longitudinally cut chromatids is observed in the upper portion of Fig. 2 *A*, and an arrow indicates the chromatid that is also shown in Figs. 2 *B–E*. An enlargement of the same section included in Fig. 2 *A* is found in Fig. 2 *B*, while the remaining panels contain the comparable region of other sections in the consecutive series. These sections were chosen because they display the typical transition for longitudinal sections as the microtome knife progresses through the chromatid. The section in Fig. 2 *B* is closest to the central axis of the chromatid and shows the greatest number of fibers that extend in the plane of the section. The fibers toward the periphery of the chromatid are seen to be radially oriented, with the suggestion of a looping arrangement. While the course of individual fibers is more difficult to distinguish toward the central axis, it is clear that the general mode of organization does not dramatically vary through the volume of the chromatid. In particular, chromosomes do not contain central holes or dense cores.

Radial, looping fibers within the plane of the section are still quite evident in panel 2 *C*, though the fibers are less densely packed than in 2 *A*. A second characteristic view of fiber arrangement, in addition to radial loops, is becoming apparent. This is a dot pattern that results from the microtome knife cutting across the radially oriented fibers. As the sections intersect the chromatid further and further from the central axis, the dot pattern is seen to replace the distribution of fibers within the plane of the section. Thus in panel 2 *D*, only a small fraction of the chromatin fibers is contained within the section. The appearance of the chromatid is now dominated by the pattern of dots. That the section is approaching the periphery of the chromatid is seen in the reduced density of dots or fibers. The final panel is very close to the outer chromatid boundary. Dots predominate, although they are sparser than in 2 *D*. The changes in the appearance of longitudinal sections as the micrographs progress through the chromatid are therefore consistent with a radial arrangement of looped fibers.

Transverse sections, which are approximately perpendicular to the chromatid axis, also provide evidence for the existence of radial loops. Fig. 3 *A* shows the typical appearance of a portion of a mitotic cell, while Fig. 3 *B* contains an enlargement of the chromosome indicated by an arrow in 3 *A*. As was observed in Fig. 2 *A*, most of the chromosomes are intersected transversely to produce stained regions of chromatin that are circular or oval. Examination of Fig. 3 *A* reveals the characteristic orientation of fibers extending from near the chromatid axis to the periphery. (It is, however, difficult to trace individual fibers for any distance.) These features of fiber distribution are observed more clearly in Fig. 3 *B*. The radial arrangement of the fibers is readily detected, and the presence of chromatin loops is strongly suggested.

Such observations are valid not only through the bodies of chromosomes but also, as revealed in Fig. 4, for the telomere regions. The four panels in the figure contain sections that progress from the body of a chromatid to its extreme tip. The stained chromatin in panel *A* is most densely packed, and both fibers and a pattern of dots are present. The dot patterns become dominant in panels *B* and *C*, and the density of material is reduced as the microtome knife exits the chromatid tip. The section in Fig. 4 *D* is barely grazing the chromatid.

Experiments were also undertaken in which mitotic

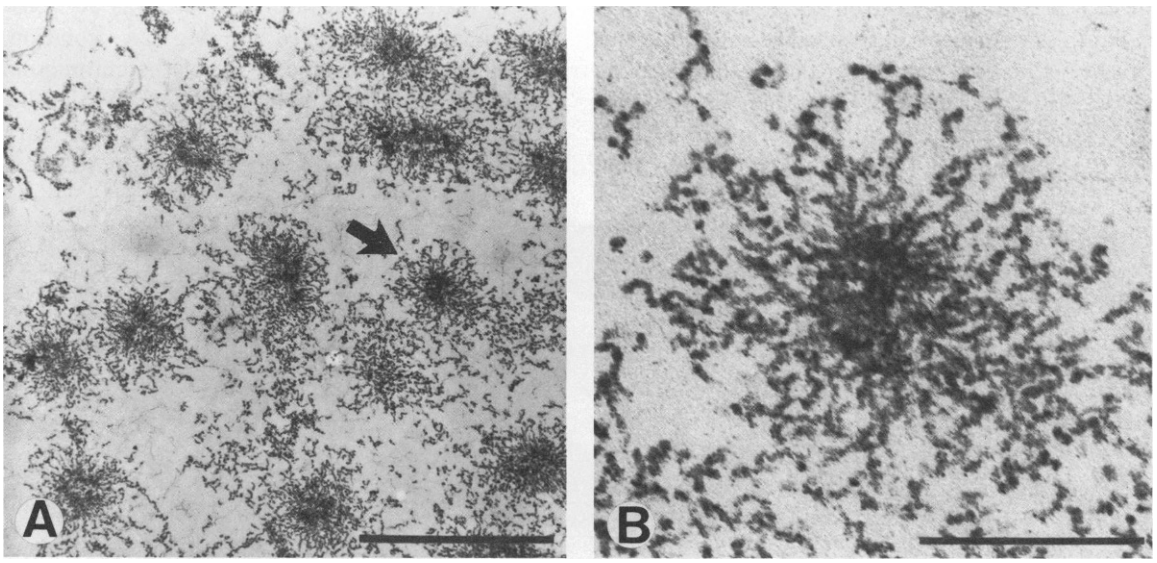

FIGURE 3 A section through a mitotic HeLa cell prepared in 1.5 mM Mg^{2+} which shows the predominantly transverse sectioning of chromosomes. Panel *A* contains a low-magnification view of a portion of a mitotic cell. An enlargement of the chromosome indicated by an arrow is included in (*B*). The radial arrangement of fibers is clearly evident. The bar represents 2.0 μm in (*A*) and 0.5 μm in (*B*).

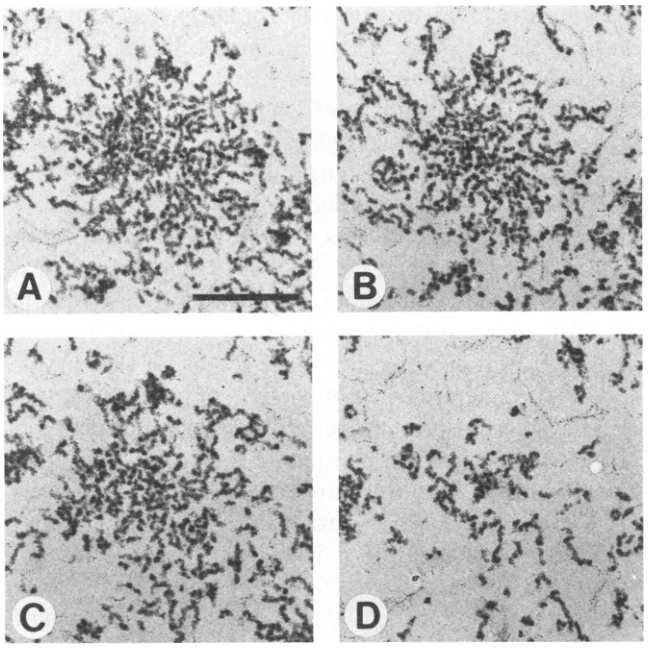

FIGURE 4 Transverse sections that graze the telomere of a metaphase chromosome. The specimen was prepared for microscopy in 1.5 mM magnesium ion. Panel *A* shows a section that is the most interior. The chromosome is barely grazed, however, in the section in panel *D*. The bar represents 0.5 μm.

HeLa cells were resuspended in hypotonic buffer containing higher and lower concentrations of magnesium ions. Thin sections of cells in 5.0 mM Mg^{2+} showed the chromosomes to be highly condensed with a largely uniform distribution of chromatin across and along the chromatid arms. Individual fibers could not be distinguished. The appearance of sections with this buffer was similar to that of mitotic cells prepared for electron microscopy in growth medium. Samples were also resuspended in buffer containing <1.5 mM magnesium ion. Under these conditions, the chromosomes further expanded and the micrographs revealed a uniform network of fibers throughout the volume of the chromatids. The orientation of individual fibers was difficult to resolve and no additional conclusions could be drawn regarding the higher-order organization of chromosomes.

Greatly expanded chromosomes with a uniform network of fibers also resulted from chelating divalent cations by resuspending samples in 5.0 mM EDTA. The general orientation of the fibers could not be readily distinguished because of the extreme degree of swelling.

Scanning EM of Chromosome Surface Structure

Scanning electron microscopy provides information regarding the surface organization of individual, isolated chromosomes, and so the results complement the conclusions obtained from transmission EM. HeLa metaphase chromosomes were prepared using two different procedures. The technique that was employed most frequently used divalent cations (Mg^{2+}, Ca^{2+}) to maintain the chromosomes in a condensed state during isolation. The magnesium ion concentration was 5.0 mM, and, as mentioned above, TEM revealed that chromosomes have a similar morphology when prepared for microscopy in 5.0 mM Mg^{2+} and in growth medium. Another protocol for chromosome isolation used polyamines (spermine, spermidine) to stabilize chromosome morphology during isolation. The results with the two procedures were generally similar, but the Mg/Ca technique was used most frequently because the degree of expansion of the chromosomes could be more reproducibly controlled.

Fig. 5 *A* displays a chromosome isolated by the spermine/spermidine procedure. The chromosome is highly condensed, and such micrographs are not very informative in revealing details of chromosome substructure. A relatively featureless surface structure was also observed for chromosomes prepared in Mg/Ca isolation buffer. Although the elongated shape of the chromosomes could be recognized, little quantitative information beyond the external dimensions could be obtained. Some variability in the appearance of chromosomes was noted between dif-

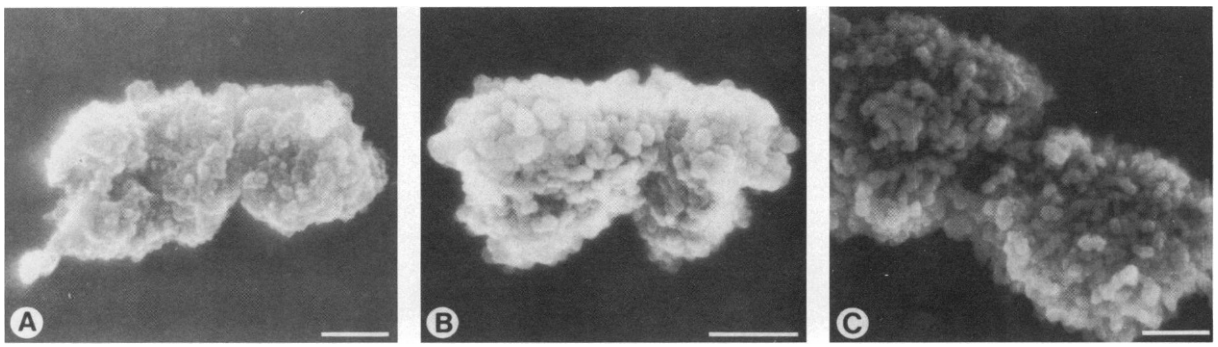

FIGURE 5 Surface structure of isolated HeLa metaphase chromosomes revealed by scanning electron microscopy. Panel *A* shows the appearance of a condensed chromosome in spermine/spermidine isolation buffer. (*B*) contains a particle isolated by the Mg/Ca procedure and resuspended in 1.5 mM Mg^{2+}. A similar specimen is shown in (*C*), except that the resuspension buffer was composed of 0.5 mM magnesium ion. The presence of knobby surface projections is apparent in panels *B* and *C*. The bar represents 0.5 μm.

ferent chromosomes and between preparations, with some particles having a rougher surface substructure. The compact morphology of the chromosomes in these solution conditions prevented the creation of deformed particles. Resuspending isolated chromosomes in reduced concentrations of divalent cations or polyamines produced a similar effect. The chromosomes expanded to produce an increase in the length and width of the particles. SEM could then be usefully applied, and the conclusions that resulted were the same for both isolation procedures.

The surface structure of isolated chromosomes showed a striking change when the magnesium concentration was decreased. At 1.5 mM, the surface was no longer relatively smooth, but consisted of well-delineated surface projections (Fig. 5 *B*). Examination of specimens in the electron microscope at different angles of tilt demonstrated that the knobby surface structure is a feature that extends uniformly around the chromatid arms. The mean diameter of the surface projections was calculated to be 69 nm. The standard deviation (SD) of these measurements was 14%. As is discussed below, the underlying chromatin fibers have a mean width of 30 nm, which is much less than the average diameter of the protuberances. An additional level of compaction of these fibers must therefore be present to account for the dimensions of the projections. Looping back of the fibers at the boundary of the chromatid would account, in part, for the 69 nm value. The resolution of the micrographs, unfortunately, is not sufficient to reveal any detailed substructure of the projections.

For chromosomes prepared in 0.5 mM Mg^{2+} (Fig. 5 *C*), the same basic structural features were present as for samples in 1.5 mM Mg^{2+}. A knobby surface structure was the dominant aspect of the micrographs. The overall dimensions of the chromosomes were enlarged and the surface density of the protuberances was reduced. Furthermore, the mean diameter of the projections decreased to 65 nm (SD = 12%). Even though the chromosomes became increasingly swollen as the magnesium ion concentration was reduced, the particles in 0.5 mM Mg^{2+} were still relatively compact. The particles did not appreciably flatten on the specimen support surface and the underlying fibers were not yet visible.

In 0.15 mM Mg^{2+}, the mean diameter of the protuberances was substantially reduced to 35 nm. Chromosomes in these conditions were partially flattened and subject to deformation.

Evidence that chromatin fibers are indeed the material composing the surface projections appeared when the magnesium ion concentration was reduced to 0.05 mM (Fig. 6 *A*, *B*). Particles in these solution conditions were extremely swollen and were consequently quite unstable. They readily flattened on the specimen support surfaces and were subject to severe distortion. But in this situation, chromatin fibers, was well as the surface projections, could be observed. The projections and fibers clearly represent different arrangements of the same material. Fibers were visible in low magnesium concentrations, which produced flattened chromosomes with fibers oriented perpendicular to the electron beam. Projections were visible in high magnesium concentrations, which produced compact chromosomes with looped fibers that were largely parallel to the electron beam. The relationship between the knobs and fibers was seen in the reduced mean diameter of the knobs (30 nm). This value now approached the width of the fibers.

The reduction in the mean diameter of the protuberances from almost 70 nm to 30 nm illustrates the important finding that the knobby surface appearance of the chromosomes was a variable feature. The factor controlling the average diameter was the degree of expansion of the chromatids.

The small value of the protuberance diameter in 0.05 mM Mg^{2+} suggested that the greater value in 1.5 mM and 0.5 mM Mg^{2+} was caused by a further compaction, perhaps supertwisting, of the loops. The substructure of the fibers may also have contributed to the surface structure of chromosomes in 0.5–1.5 mM Mg^{2+}. The fibers were not smooth (Fig. 6 *B*), but appeared as stacks of disks. The

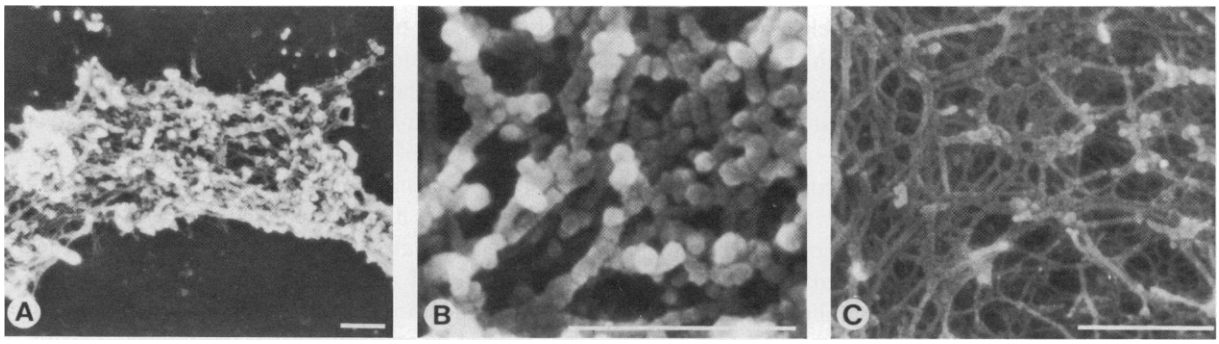

FIGURE 6 Scanning EMs of highly swollen chromosomes resuspended in buffer containing low concentrations of magnesium ion. The chromosomes were purified using the Mg/Ca technique. The chromosomes in (*A*) and (*B*) were in 0.05 mM Mg^{2+}. The underlying fibers as well as the surface protuberances are visible. The effect of high salt concentrations is demonstrated in panel *C*, which displays a chromosome in 1.5 mM Mg^{2+} with 1.0 NaCl added. The bar represents 0.5 μm.

periodicity between disks was 15 nm, and so these may represent turns of the helix of the 10 nm nucleosomal filament.

Decreasing the magnesium ion concentration below 0.05 mM to 0.015 mM expanded the chromosomes even further, but did not reduce the width of the chromatin fibers. It was necessary to include high concentrations of monovalent salt (NaCl) to destabilize the fibers. Fig. 6 *C* shows the completely flattened network of filaments that resulted from adding 1.0 M NaCl to a chromosome suspension in 1.5 mM Mg^{2+}. The filaments have a width of 8.2 nm and display the result of partially extracting histones to produce narrow, unstable DNA-histone filaments.

DISCUSSION

Transmission EM and scanning EM observations were correlated to determine how chromatin fibers are assembled into the characteristic morphology of metaphase chromosomes. A similar experimental approach was used with both techniques to swell the chromosomes slightly and thereby separate the fibers. TEM of serial sections of particles in 1.5 mM Mg^{2+} yielded the strongest evidence that fiber distribution is predominantly radial both across and along each chromatid. This observation was supported by the resolution, using SEM, of knobby surface projections on isolated chromosomes. Adjusting the Mg^{2+} concentration revealed that the protuberances are a variable feature, because their diameters ranged from 30 nm to 70 nm.

An advantage of using such a protocol was that histones and other proteins were not extracted. Another advantage was that the presence of Mg ions served to stabilize the nucleosomal substructure of the fibers. The expansion of chromosomes could also be readily reversed by increasing the magnesium ion concentration. Furthermore, the particles were not treated with enzymes to uncover chromosomal substructure. Thus the observations did not result from the use of harsh experimental procedures.

The two EM techniques provided compelling evidence that the chromatin fibers of metaphase chromosomes have a fundamentally radial distribution, with the fibers organized as loops. Essentially a single fiber is folded into each chromatid, so that the radial fibers must loop back at the chromatid periphery.

How different loops are connected could not be determined. Even though the peripheral fibers were separated in 1.5 mM Mg^{2+}, the central region of each chromatid was still densely packed with chromatin and the bases of the loops were not resolved. Another complication is the size and complexity of eukaryotic chromosomes. The great size of these chromosomes produced distortion of the native structure of the particles during processing for EM. Probably as a consequence of applying this procedure, the surface protuberances seen by SEM showed no evidence of a regular arrangement along the length of the chromatid. The dot patterns on longitudinal thin sections observed by TEM also revealed no obvious order.

Some discrepancies between the scanning EM data and the transmission EM data are apparent. The radial fibers observed with transverse thin sections of specimens in 1.5 mM Mg^{2+} (Fig. 3) would seem to comprise more open loops than the condensed fibers which must underlie the 69 nm surface protuberances (Fig. 5 *B*). Part of the reason for this discrepancy is that the knobby surface appearance of isolated chromosomes was exaggerated by the thickness (8 nm) of the gold/palladium coating. The vaporized metal would tend to accumulate on the protruding tips of the loops, thereby giving the tips a more bulbous appearance than actually exists.

Another possible source of discrepancy is that chromosomes were prepared for SEM and TEM using different procedures. Somewhat different degrees of expansion of the chromosomes could have resulted in loops that were compacted to variable extents. The effect of magnesium ions in controlling chromosome swelling was quite reproducible, but further processing of the samples involved dehydration steps using organic solvents followed by critical point drying (for SEM) or embedding in plastic (for TEM). Even though specimens were fixed with glutaraldehyde, shrinkage of chromosomes clearly occurred in the organic solvents. Different extents of chromosome shrinkage with the two EM procedures could create the more condensed loops recorded by SEM. This would not diminish the significance of the results, because the same structural changes were found by adjusting the magnesium ion concentration from high-to-low values. But the appearance of looped fibers for a particular Mg^{2+} concentration would only be expected to be similar in comparing the TEM and SEM results, and not precisely identical.

Shrinkage of specimens may also account for the difficulty in observing radial loops by SEM along flattened chromosomes in low magnesium concentrations (Fig. 6 *A*, *B*). Chromosomes were allowed to adhere to glass specimen support surfaces before the specimens were dehydrated. Consequently, the particles were probably distorted as the bulk of the chromosomes shrunk in the organic solvents, except where chromatin fibers were anchored to the specimen supports. Whether improvements in specimen preparation techniques could surmount the problem of chromosome distortion remains to be resolved. Substantial deformation may be inevitable because of the highly expanded state of the chromosomes and because flattening on the support surface cannot be avoided. TEM holds an advantage over SEM in this regard. The three-dimensional shape of chromosomes is not altered by embedding the specimens in plastic.

The thin sectioning and SEM results suggest that the predominant mode of fiber organization is a radial loop arrangement. But longitudinal fibers were detected by SEM for chromosomes in 0.05 mM Mg^{2+} (Fig. 6 *A*).

Longitudinal fibers may have been produced by shrinkage of chromosomes during processing for EM. The particles have certainly been considerably distorted. A predominant organization as radial loops would not, however, discount the possibility of longitudinal fibers being present. Such fibers were not visible in thin sections, which are less deformed, but a minority of longitudinal fibers could be masked by the radial loops. Longitudinal fibers would most likely be present close to the chromatid axis. The fibers would probably be composed of the same DNA-histone material as the 30 nm filaments of the loops. They would not constitute a separate "core" formed from special nonhistones.

These ideas about metaphase chromosome organization are schematically illustrated in Fig. 7. Chromatin loops that are connected to form a continuous fiber are shown in Fig. 7 *A*. A very open arrangement of loops is depicted, but the regions near the bases of the loops could be much more closely associated and overlapping of the fiber could result. As illustrated in Fig. 7 *B*, the main portion of each loop might be supertwisted to create a more compact and elongated segment of chromatin. Supertwisting could account for the knobby appearance of the surfaces of isolated chromosomes in 0.5–1.5 mM Mg^{2+}. The degree of supertwisting could be Mg^{2+}-dependent, which would explain the variability in the average diameters of the surface protuberances. In Fig. 7 *C*, loops are shown to progress along the direction of the chromatid axis by being arranged on a shallow helix. No experimental evidence exists for this type of ordered distribution of loops, but a helical arrangement might facilitate assembly of chromatin fibers into metaphase chromosomes. Longitudinal fibers are also depicted as a distinct mode of chromatin fiber organization. These fibers could serve to connect different portions of the chromatid.

Whether the same chromatin segments are found in the same loops during each mitosis is not known. A specific nucleotide sequence might be present at the base of each loop, and this sequence may be responsible for bringing the bases of the loops into close proximity during mitosis. This possibility would mean that the same loops are formed during each mitosis. More concrete evidence in favor of specific loops is the formation of chromosome banding patterns (G-bands, R-bands, etc.). The reproducibility of these patterns argues that chromosome substructure is conserved from one cell cycle to the next.

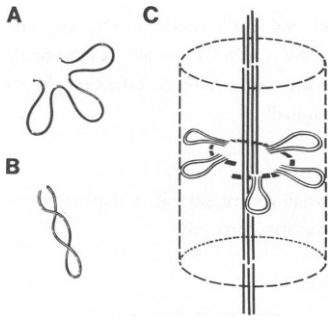

FIGURE 7 Drawing showing the organization of 30 nm fibers into loops (*A*, *B*) and the possible arrangement of chromatin loops along a helix (*C*). The axis of the shallow helix is coincident with the chromatid axis. This hypothetical, symmetric arrangement of chromatin fibers would facilitate assembly of metaphase chromosomes as the cell cycle progresses from interphase to mitosis. Longitudinal fibers, which are parallel to the chromatid axis, are also shown to be present.

Fig. 7 shows the chromatin loops to be approximately the same size, but some variability may occur in the amount of DNA in each loop. Measurements of the contour lengths of DNA loops were obtained for histone-depleted chromosomes (23). The range from 10–30 μm (30–90 kb) suggests that chromatin loops in intact chromosomes possess considerable variation in their contour lengths. However, the substantial variation for histone-depleted chromosomes may have been due to incomplete removal of histones.

All these EM results have been for metaphase chromosomes, but the organization of the genome into chromatin loops is of greater functional significance during interphase. DNA replication and gene transcription are two nuclear functions that are regulated by the organization of the genome into chromatin loops. DNA loop length during interphase was shown to average ~85 kb, with the range of values reported by a number of laboratories between 75–220 kb (1–3). The looping pattern may therefore be conserved from interphase to metaphase, as suggested by the similarities of loop lengths for the two phases of the cell cycle.

The combination of transmission EM and scanning EM, along with the use of appropriate buffers to separate chromatin fibers, has provided a potent approach to understanding metaphase chromosome architecture. The significant information that has been obtained is a stimulus to extend the EM methodology to obtain a more detailed picture of chromosome organization.

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DISCUSSION

Session Chairman: Lee Makowski
Scribes: Robert Bruccoleri and Ian Armitage

LANGMORE: You state that you do not use any harsh preparative procedures. Could you please comment on the large body of x-ray diffraction evidence going back to the studies by Nicolaeff, Pooley et al., and that Jim Paulson and I did, that glutaraldehyde-fixed chromatin loses its native internal structure when prepared in organic solvents such as ethanol?

ADOLPH: This is secondary to the subject of our paper because we were not concerned with the internal structure of the fibers. Historically, there have been many observations of the higher-order arrangement of 30-nm fibers, and many of them could have been affected by the preparative procedures. It's still not resolved in many cases what the effect of the preparative procedures is. For example, various organic solvents (ethanol, methanol, acetic acid) have been used in sample preparation for many years, with both light and electron microscope observations of metaphase chromosomes. An early example is the observation of coiled chromatids in either low pH or methanol/acetic acid preparations of chromosomes, which is contradictory to our observation of direct condensation of loops. But one doesn't know how much the treatments would extract histones or affect the higher order structure. We don't believe that any disruption in fiber substructure would be seen at the level we're observing, namely the orientation of the fibers in the chromosome. As we extend our observations to higher and higher resolution, then the internal structure of the fibers becomes more pertinent. We'd like to ask, are the loops organized along the chromatid in any sort of order? In our paper, we don't report seeing any such order, but that would be something we would like to see. It may be that the preparative procedures that we are using may be affecting the order in which the radial loops are arranged.

MAKOWSKI: So what you're saying is that radial organization is not changed by the preparative procedure.

ADOLPH: I doubt that major conclusions of our paper are affected. As we probe into the substructure, future progress will depend on the preparative procedures.

LANGMORE: Can you cite any evidence from light microscopy that the structures you're seeing are native?

ADOLPH: Light microscopy does not provide sufficient resolution to see the fibers. We can say that the chromosomes in the electron microscope look healthy, neither stretched nor abnormally condensed, nor is there any adhering material.

DURKIN: Your SEM micrographs show knobs that you are interpreting as radial loops. As you go to lower concentrations of Mg^{++}, the knobs shrink until at 50 μm Mg^{++}, you have 30-nm knobs. I don't understand how the underlying fiber, which has a diameter of 30 nm, can loop back, be covered with metal, and still have a diameter of 30 nm.

ADOLPH: Those dimensions are quite approximate. One sees a decrease in the size of the knobs from more than twice the fundamental 30-nm fiber down to $\sim$ 30 nm. One could speculate that this represents the unfolding of a supertwisted loop and that the fibers seen at 50 μm Mg^{++} do not loop back in close proximity. It is clearly a direction for future research because we are not resolving structure down to the level of the nucleosomes.