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RELATIONSHIP OF THE SURFACE STRUCTURE OF METAPHASE CHROMOSOMES TO THE HIGHER ORDER ORGANIZATION OF CHROMATIN FIBERS

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Abstract

Scanning electron microscopy (SEM), as well as transmission electron microscopy (TEM), has been utilized to determine how the surface structure of mitotic chromosomes is related to the organization of the 30 nm chromosomal fibers. SEM revealed the surfaces of isolated, HeLa cell chromosomes to possess a knobby substructure with chromosomes prepared for EM in buffers containing 0.5-1.5 mM Mg^{2+}. These projections had substantially greater widths (65-70 nm) than the underlying chromatin fibers. Reducing the Mg ion concentration to 0.05-0.15 mM resulted in the further expansion of the chromosomes, which flattened the chromosomes for SEM so the fibers became the dominant feature of the micrographs. The surface protuberances are interpreted as representing the peripheral tips of radial chromatin loops.

The same procedure of slightly expanding chromosomes by decreasing the Mg^{2+} concentration in resuspension buffer was also utilized in a TEM, serial sectioning study. Longitudinal sections close to the central chromatid axis showed radially oriented fibers within the planes of the sections. This was replaced by a dot pattern when the longitudinal sections grazed the periphery of the chromatid. Transverse sections displayed more clearly the radial orientation of the fibers.

A consistent picture emerges from applying SEM and TEM that supports the "radial loop" model for the primary mode of organization of chromatin fibers in metaphase chromosomes.

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Introduction

SEM, in addition to TEM, has been applied for many years to the problem of determining the path of folding of the fundamental chromatin fibers in mitotic chromosomes. Since the mid-1970s these investigations have received a new impetus from the discovery that the substructure of chromatin fibers consists of octamers of the core histones H2A, H2B, H3 and H4 interacting with DNA to form nucleosomes [24]. However, the higher-order organization of nucleosomes in chromatin fibers and the mode of packaging the fibers in mitotic chromosomes remain incompletely understood. Electron microscopy of isolated chromatin and thin sectioning investigations of intact nuclei and metaphase chromosomes have established that the primary chromatin fibers have widths of 10 nm and 20-30 nm [13,15,28,12,27,30,25]. The 10 nm fibers correspond to the beads-on-a-string nucleosome filament, and models have been presented, based mainly on EM data and some physical chemical results, for the folding of this filament into the 20-30 nm fibers. A helical mode of folding [14] has gained the widest acceptance, though experimental evidence for the compaction of nucleosomes into "superbeads" has been published [20]. Helical fibers could be created either by directly coiling the 10 nm nucleosomal filaments [14] or by coiling a zig-zag ribbon of nucleosomes [32].

SEM has been employed in a large number of investigations to determine the substructure of metaphase chromosomes [9,16,8,11,21,22,3,19, 17,18,5]. Using the technique allowed aspects of chromosome substructure to be investigated that include chromatin fiber arrangement, chromosome banding patterns, secondary constrictions and induced lesions. Only recently, however, has the resolution of detail been sufficient to reveal information regarding the organization of the underlying chromatin fibers. The use of an osmium impregnation technique by Allen and colleagues has provided a high-resolution picture of chromosome surface structure [19,17,18]. The purpose of this paper is to relate the results from SEM to the view of chromatin fiber arrangement given by TEM serial sections [4,5]. A study of histone-depleted HeLa metaphase

chromosomes suggested that the DNA strands are primarily arranged as radial loops [6,26]. Correlating the SEM and TEM data is shown to support the "radial loop" model of chromosome structure. The experimental approach with the two EM techniques was similar: chromosomes were expanded by reducing the divalent cation (Mg^{2+}) concentration, which separated the fibers and thereby allowed both the surface structure (SEM) and internal arrangement (TEM) of the fibers to be observed.

Materials and Methods

Preparation of Mitotic Chromosomes

HeLa S3 cells were propagated in suspension culture using minimum essential medium (GIBCO; Joklik modified for suspension culture) containing 5% fetal bovine serum (Flow).

Mitotic chromosomes were purified by two different procedures, one of which employed divalent cations (Mg^{2+}, Ca^{2+}) to maintain the chromosomes in a condensed state during isolation while the other used polyamines (spermine, spermidine) [1]. For the SEM experiments, reducing the Mg^{2+} concentration was found to be most effective for reproducibly expanding the isolated chromosomes, and so samples were usually prepared by the divalent cation procedure. A high proportion of mitotic cells was obtained by treating cultures with colchicine (0.2 μg/ml) for 12-16 hr. The treated cells were cooled on ice, and all subsequent steps in the isolation procedure were performed at 4°C. Cells were resuspended in isolation buffer (50 mM NaCl, 5 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM PMSF (phenylmethylsulfonyl fluoride)) and disrupted in a Dounce tissue homogenizer after adding NP40 detergent (0.5%) and sodium deoxycholate (0.1%). For the polyamine isolation procedure, the 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, 0.1 mM PMSF. After low speed centrifugation to remove nuclei and large debris, chromosomes were pelleted through 40% sucrose in isolation buffer containing 0.5% NP40.

Processing of Samples for Scanning Electron Microscopy

Chromosomes were expanded from their compact state in Mg/Ca isolation buffer by resuspending the particles in a selected concentration of $MgCl_2$ (1.5, 0.5, 0.15 or 0.05 mM), buffered with 5 mM Hepes, pH 7.4. The resuspended chromosomes were placed on glass coverslides in a 4°C cold room, and, after 30 min, the coverslides were covered with 0.8% glutaraldehyde in the appropriate buffer. Fixation was allowed to proceed for 60 min, after which the glass slides were washed with buffer and the chromosomes were treated with 0.05% osmium tetroxide for 30 min. The slides were again washed with buffer. The samples were dehydrated through ethanol (25, 50, 75 and 100%) at 4°C, transferred to 50% ethanol, 50% Freon 113, and then to 100% Freon 113 before critical-point drying with liquid CO_2. Dehydration with acetone produced similar SEM results.

Processing of Samples for Transmission Electron Microscopy

Mitotic HeLa cells, prepared by treating cultures with colchicine, were resuspended in 10 mM NaCl, 10 mM sodium cacodylate, pH 7.0, 1.5 mM $MgCl_2$, 0.1 mM PMSF. NP40 detergent was added to a concentration of 0.1% for 30 min, following which the cells were mixed with glutaraldehyde to give a final concentration of 0.8%. After 90 min, the samples were washed with 0.1 M sodium cacodylate, pH 7.0, and treated with 1% osmium tetroxide (in 0.1 M sodium cacodylate, pH 7.0) for 90 min. The cells were washed with buffer and then immobilized by pelleting through molten 2% agarose.

Small blocks (1-2 mm square) of cells in agarose were pre-stained with 1% uranyl acetate for 20 min before being dehydrated through acetone (25, 50, 75 and 100%). The material was finally embedded in Epon 812 for sectioning.

Electron Microscopy

For SEM, critical-point dried chromosomes were coated with gold and palladium. A Denton apparatus was used to vaporize equal amounts of gold (100%) wire and gold/palladium (60%/40%) wire. Latex spheres with diameters of 109 nm were included to measure the thickness of the coating, which was about 8 nm. The scanning mode of a JEOL JEM-100CX electron microscope was employed to examine and photograph the specimens. Most photographs were taken at 40 kV.

Epon-embedded mitotic cells were serially sectioned for TEM with a diamond knife. The sections (silver-gray, about 60 nm) were mounted on carbon-coated formvar support grids and stained with 1% uranyl acetate and lead citrate. A Hitachi HU-11C electron microscope was used to obtain the micrographs at 100 kV.

Results

SEM Observations of the Surface Structure of Chromosomes

HeLa mitotic chromosomes were prepared in buffers containing either divalent cations or polyamines to maintain a condensed morphology during isolation. Employing these buffer conditions was essential to avoid stretching the particles as the mitotic cells were disrupted. SEM of individual chromosomes in isolation buffer confirmed the light microscope observations in revealing a relatively smooth surface appearance without definable substructure. Figure 1 shows a mitotic chromosome in isolation buffer containing divalent cations (5.0 mM Mg^{2+}, 0.5 mM Ca^{2+}), and Figure 2 displays a chromosome isolated in polyamine containing buffer (0.5 mM spermine, 0.15 mM spermidine). While the characteristic metaphase chromosome morphology is recognizable in both micrographs, no conclusions can be made regarding the organization of the underlying chromatin fibers.

A striking change in the surface appearance of the particles became apparent upon resuspending the chromosomes in buffers containing a reduced concentration of Mg ions
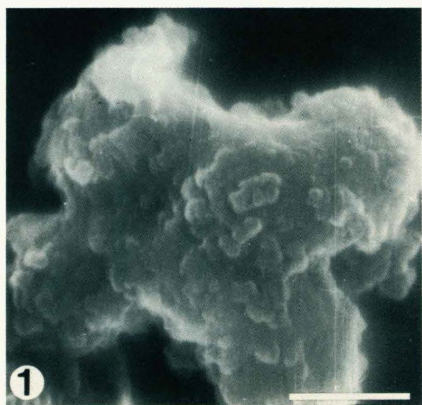
Figure 1. HeLa mitotic chromosome isolated in buffer containing divalent cations (5.0 mM Mg^{2+}, 0.5 mM Ca^{2+}) and prepared for scanning electron microscopy in this buffer. A relatively smooth surface structure is observed. Bar = 0.5 μm.
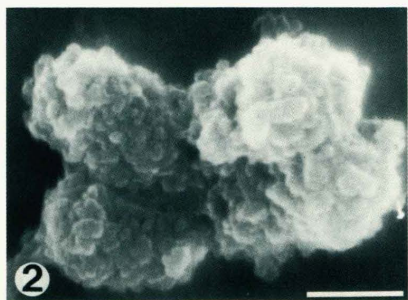

Figure 2. HeLa mitotic chromosome isolated and prepared for SEM in polyamine-containing buffer. Bar = 0.5 μm.

before preparing the samples for SEM. (The same change was detected by decreasing the polyamine concentration, but the results were less reproducible than by adjusting the Mg^{2+} concentration.) A chromosome prepared in 1.5 mM Mg^{2+} is shown in Fig. 3. The prominent features of the micrograph are the knobby projections on the surface of this expanded chromosome. The surface protuberances have a diameter of approximately 69 nm, with a standard deviation of $\pm14\%$. The structure of the particles is similar with the Mg ion concentration further reduced to 0.5 mM (Fig. 4). The projections have a slightly smaller mean diameter for these conditions of 65 nm ($\pm12\%$ SD). These dimensions are greater than the width of the basic chromatin fibers (30 nm), and therefore appear to reflect a level of further compaction or supertwisting of the fibers.
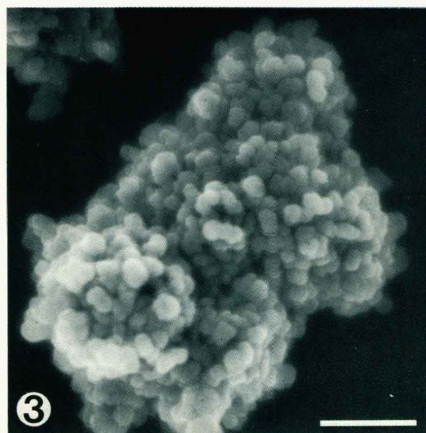

Figure 3. Chromosome isolated in buffer containing divalent cations and resuspended in a Mg^{2+} concentration of 1.5 mM. Surface protuberances, having diameters of almost 70 nm, are evident. Bar = 0.5 μm.
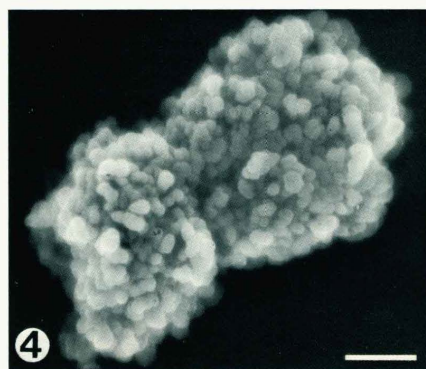

Figure 4. Chromosome prepared for SEM in 0.5 mM Mg^{2+}. The projections have diameters of 65 nm. Bar = 0.5 μm.

To relate the surface appearance of the chromosomes to the arrangement of the chromatin fibers, the Mg^{2+} concentration of the resuspension buffer was lowered to 0.15 mM (Fig. 5) and 0.05 mM (Fig. 6). For this range of concentrations, the overall dimensions of the chromosomes continue to increase and this expansion is accompanied by a decrease in the diameters of the projections and the number per unit area. The protuberances possess mean diameters of 35 nm in 0.15 mM Mg^{2+} and 30 nm in 0.05 mM Mg^{2+}. The chromosomes have expanded to such an extent, particularly in 0.05 mM Mg^{2+}, that the particles flatten on the glass coverslides during preparation for SEM. Because

of flattening, the chromatin fibers become more noticeable, but the chromosomes suffer from greater distortion. This makes tracing individual fibers very difficult for these complex chromosomes. To overcome the problem, TEM of serial sections was utilized, as described in the following section. Fig. 5 demonstrates that chromosomes in 0.15 mM Mg^{2+} are only partially destabilized, since one portion of the chromosome shows a knobby surface structure, while stretched fibers are visible in another portion. But, as seen in Fig. 6 (which may contain more than one chromosome), the degree of flattening is more pronounced for specimens in 0.05 mM Mg^{2+}. Although longitudinal bundles of fibers are detected, the distortion of the particles prevents any conclusions to be made concerning the in situ significance of longitudinal fibers. Figure 7 shows a part of a chromosome in 0.05 mM Mg^{2+} at a higher magnification. The chromatin fibers have an uneven substructure, which may reflect the superhelical organization of the 10 nm nucleosome filament. The important result of these experiments is that the knobby surface substructure in 0.5-1.5 mM Mg^{2+} is directly related to the underlying chromatin fibers, observed most readily in 0.05-0.15 mM Mg^{2+}, since a smooth transition occurs between the two types of chromosomal substructure.

Chromosomes were further destabilized by including 1.0 M sodium chloride in the resuspension buffer to partially extract histones (Fig. 8). The particle is completely flattened and consists of a tangle of 8 nm fibers.

TEM Observations of Serial Sections of Chromosomes

The approach of reducing the Mg^{2+} ion concentration to slightly expand the chromosomes and thereby separate the chromatin fibers was also followed in a TEM study of serial sections. It was not necessary to use isolated chromosomes, and so intact mitotic cells were resuspended in an appropriate buffer before processing for TEM. Samples prepared in 5.0 mM Mg^{2+} showed a uniformly dense distribution of stain across and along the chromatid arms. Individual strands of chromatin could not be resolved, which is compatible with the SEM results. But the overall dimensions of specimens in 1.0-1.5 mM Mg^{2+} had increased by 30%, and this allowed separate strands to be distinguished. Figure 9 shows the typical appearance of a mitotic HeLa cell prepared in a buffer containing 1.5 mM Mg^{2+}. The chromosomes are well-defined because the buffer also contained NP40 detergent to extract most of the membranous material in the cell. Chromosomes are mainly sectioned transversely to the chromatid arms because the chromatids are considerably longer (2-10 μm) than they are wide (1 μm).

Longitudinal sections, cut parallel to the chromatid arms, are rarer, but examples are presented in Fig. 10. Both sister chromatids are encountered by the sectioning knife in the figure. The fibers are generally seen to be oriented radially to the chromatid axes, though the fibers twist from the plane of the section

and cannot be followed for any distance. The transition from a central, longitudinal section to a peripheral section is revealed in the four sections. Examination of the four sections and others in this series of micrographs demonstrates that sections which graze chromatids primarily show a dot pattern. This is interpreted as resulting from the sectioning knife cutting across the radially arranged fibers. But as the sections penetrate the chromatid, the array of dots is superseded by fibers which lie within the section and which are oriented away from the central chromatid axis. Longitudinal fibers which parallel the axis may be present, but the predominant arrangement is radial.

Sections which cut across the chromatids also display the radial orientation of the fibers (Fig. 11). This consecutive series of transverse sections shows a similar pattern for each member of the series which consists of fibers extending from near the center to the outer region of the chromatid. Essentially one, long chromatin fiber is packaged in each chromatid, so that the radial fibers must loop back at the periphery. Complete loops are infrequently visible, however, because of twisting of the fibers from the section. (The thickness of the sections is about twice that of the fiber diameter.)

The structure of telomeres was also investigated. Grazing sections that penetrate the tip of a chromatid show the transition from a dot pattern to a distribution of fibers within the section. This transition was characteristic of grazing, longitudinal sections, and so the results demonstrate that telomeres have a structure similar to the rest of the chromosome periphery.

Discussion

The aim of this investigation was to correlate SEM and TEM observations of HeLa mitotic chromosomes and thereby relate the surface structure of chromosomes to the distribution of the 30 nm chromatin fibers. The experimental approach was the same for both EM procedures. Decreasing the concentration of divalent cations in resuspension buffer expanded the chromosomes and separated the 30 nm fibers. Employing this technique had a number of advantages. Proteins were not extracted or digested and the expansion was reversible. The nucleosome substructure of the fibers was not altered since nucleosomes were stabilized by the Mg ions. The experiments revealed that the knobby surface projections of isolated chromosomes, seen most clearly for Mg^{2+} concentrations of 0.5-1.5 mM, are a variable feature that represent the peripheral tips of radially oriented fibers. The diameters of the protuberances (close to 70 nm in 1.5 mM Mg^{2+}) suggest a further compaction or supertwisting of the 30 nm fibers.

The micrographs of slightly swollen chromosomes are strong evidence that the basic mode of organization of chromatin fibers during mitosis is a radial distribution. Complete loops were difficult to follow in the TEMs of thin

Figure 5. The chromosome, swollen by resuspension in 0.15 mM Mg^{2+}, partially reveals the underlying 30 nm fibers. Bar = 0.5 μm.
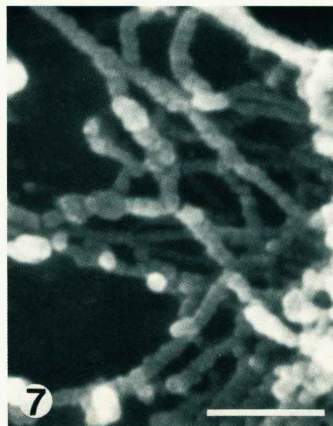

Figure 7. Higher magnification view of a portion of a chromosome prepared in 0.05 mM Mg^{2+}. Bar = 0.25 μm.
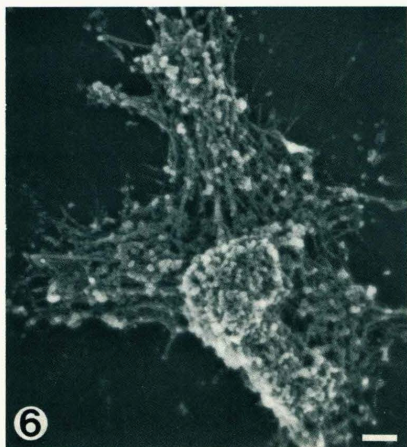

Figure 6. Fibers are the dominant feature of the chromosome, which was resuspended in 0.05 mM Mg^{2+} for SEM. Bar = 0.5 μm.
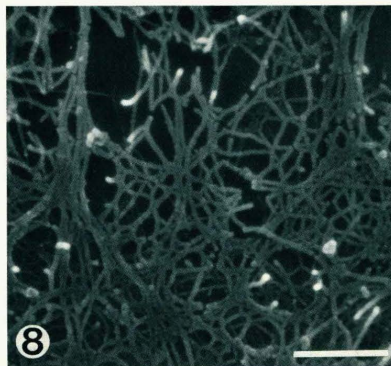

Figure 8. Including 1.0 M NaCl, along with 1.5 mM Mg^{2+}, in the buffer extracts histones and produces a complete flattening of the chromosome. Bar = 0.5 μm.

sections, but the existence of radial loops is inferred from the fact that each chromatid consists of essentially a single fiber which is repetitively folded to create the metaphase chromosome morphology. The SEM and TEM evidence for radial loops is convincing, but leaves a number of fundamental questions unanswered. How much DNA is present in each loop? Are the loops specific? Do longitudinal fibers exist? How are the loops connected? What is the relationship of the metaphase chromosome loops to the loops of interphase nuclei?

The distribution of DNA lengths in loops of intact chromosomes would appear to be the same as found for histone-depleted chromosomes [26]. Measurements from micrographs of histone-depleted, HeLa chromosomes showed the contour lengths to range from 10-30 μm (30-90 kb), with an average of 23 μm (70 kb) with respect to the amount of DNA in a loop. The DNA loops are compacted 40-fold upon interacting with histones to form nucleosomes. It is unclear whether the distribution of DNA lengths is due to the in situ variability of chromatin loop sizes or is due to incomplete unfolding of the DNA upon extracting histones.

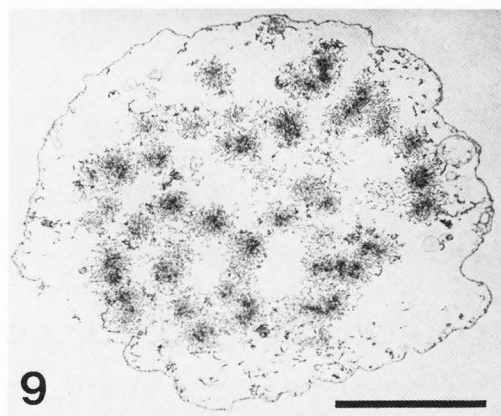It would be of interest to determine if the

Figure 9. Transmission electron micrograph of a section through a mitotic HeLa cell. The cell was resuspended in hypotonic buffer containing NP40 detergent, and the chromosomes were slightly expanded by adjusting the Mg ion concentration to 1.5 mM. Bar = 0.5 μm.
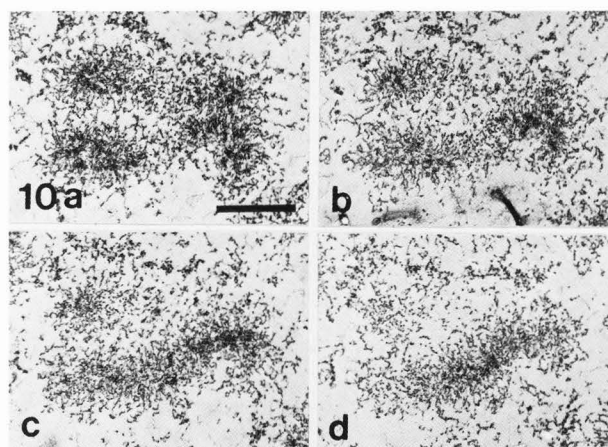

Figure 10. Longitudinal sections that display the transition from a dot pattern as the sections first penetrate the chromatids to a pattern of fibers in the plane of the section. Bar = 1.0 μm.
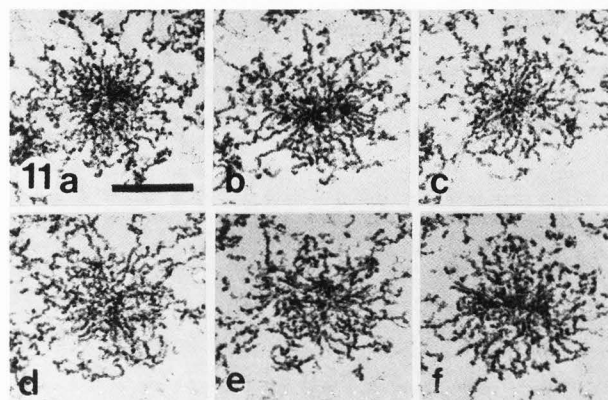

Figure 11. Transverse sections through a chromosome prepared in 1.5 mM Mg^{2+}. These consecutive sections clearly demonstrate the radial arrangement of the fibers. Bar = 0.5 μm.

loops are specific. That is, are the same segments of DNA found in the same loops at mitosis of each cell division cycle? No direct evidence is available, but the formation of reproducible chromosome banding patterns suggests that the substructure of chromosomes is accurately preserved from mitosis to mitosis. The preservation of specific loops, which may originate from features of the DNA nucleotide sequence, is therefore likely to occur.

EM shows that the bulk of chromatin fibers of HeLa metaphase chromosomes are oriented radially around the chromatid axes, but longitudinal fibers could also be present. Firm conclusions are difficult to reach from SEMs of greatly expanded chromosomes, which clearly reveal the fibers but which are usually severely distorted. The most informative TEMs were from chromosomes in 1.0-1.5 mM Mg^{2+}. But the region near the chromatid axes remained highly condensed in these conditions, so that longitudinal fibers could not be discerned. Decreasing the Mg^{2+} concentration resulted in the chromosomes opening into a network of fibers in which longitudinal fibers were not apparent. Thus the presence of fibers close to and paralleling the chromatid axes remains speculative. Such fibers could have important roles in connecting loops that are adjacent or are separated by a substantial fraction of the length of the chromatid.

Unravelling how different loops are connected is difficult, since the bases of the loops originate near the densely-packed centers of the chromatids. Developing a complete model of metaphase chromosome structure requires knowing whether packaging of the loops is ordered. A regular arrangement of structural units is observed for many biological systems. Such regularity provides an efficient mechanism for assembling and maintaining the structure. For chromosomes, the loops could be arranged along a shallow helix whose axis is the chromatid axis. Unfortunately, HeLa chromosomes, and the chromosomes of other higher eukaryotes, are too complex and prone to distortion during preparation for EM to allow any regularity of structure to be observed. Improving the techniques for specimen preparation and microscopy to increase the possibility of observing an ordered arrangement of loops will have a high priority in further experiments.

This EM investigation has been exclusively concerned with metaphase chromosomes, but the significance of DNA loops in interphase nuclei has been recognized in recent years. Loops of DNA, with the bases of the loops immobilized in the residual protein skeleton of the nucleus, were uncovered by extracting histones and other proteins under mild conditions. The apparatus of DNA synthesis was demonstrated to be associated with the bases of the loops as components of the nuclear "matrix" or "cage" [23,31]. RNA

transcription was also shown to be related to the looped arrangement of the DNA, since specific genes were located at defined positions with respect to the loops [10,29]. From a number of investigations, the DNA domain size for interphase chromatin was found to be approximately 75-220 kb. Lengths of about 85 kb are most frequent. These values for the lengths of the DNA domains in interphase are similar to the sizes in metaphase, which range up to 90 kb. The similarity of the values may imply that the same pattern of looping is conserved throughout the cell cycle. However, the proteins that are involved in anchoring the loops appear to be different. The structure of the histone-depleted nuclei is primarily maintained by the "lamins", which are not associated with mitotic chromosomes [2].

The "radial loop" model of metaphase chromosome structure that is derived from the SEM and TEM results shares some of the basic features of the "folded-fiber" model [13]. Both recognize that the 30 nm fibers are directly folded into the metaphase chromosome morphology, with no intermediate levels of organization. The EM results with intact chromosomes are also compatible with the micrographs of histone-depleted chromosomes, which strikingly demonstrate the existence of DNA loops [26]. However, very different models of chromosome structure have been presented. In particular, the "supersolenoid" model considers that a hierarchy of levels of coiling exists between the 30 nm fibers and completed chromosomes [7,30].

These results show that SEM is a powerful technique that can be profitably applied to the problem of metaphase chromosome substructure. The challenge for further SEM studies of chromosome organization is to improve the resolution of detail to observe the organization of nucleosomes along chromatin fibers and the arrangement of looped fibers along the chromatid arms.

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Discussion with Reviewers

Reviewer I: The major conclusions of your research are that the protuberances on the chromosome surface seen by SEM represent the tips of radial loops and that the serial sections studied by TEM confirm the radial loop model of metaphase chromosome structure. Would you please place these results in the perspective of what is in the literature?

Authors: SEM has been applied since the 1960s to the problem of understanding the structure of metaphase chromosomes. In the early studies, SEM provided results that were less revealing than TEM of whole mount specimens. The external outlines of chromosomes could be recognized by SEM, but details of surface substructure could not be resolved (text references 8,9,16). Improvements in specimen preparation techniques and microscopy allowed "microconvules" to be visualized on chromosome surfaces (text reference 11). But the relationship between these surface features and the underlying organization of the fundamental chromatin fibers was not clear. The experimental approach followed in this paper was to slightly expand chromosomes without extracting proteins and to correlate the results of both SEM and TEM observations. This approach had not previously been reported in the literature. The conclusion that the surface protuberances represent the peripheral tips of radial chromatin loops has been supported by other SEM and TEM investigations (e.g., text reference 22). High resolution SEM procedures have supplied Allen and colleagues (text references 17-19) with an unprecedented, detailed view of chromosome surface topography. But these workers were more concerned with cytogenetic questions and not with the structural problem of relating the surface appearance to the arrangement of the 30 nm chromosomal fibers.

Reviewer III: Would you comment on the technical limitations of your SEM procedure for interpretation of the images? In particular, does the thickness of the Au/Pd coating of the chromosomes allow observation of the details intended?

Authors: The purpose of these experiments was to uncover information regarding the general path of folding of the 30 nm chromosomal fibers in metaphase chromosomes. The significant technical innovation was to resuspend isolated chromosomes in the buffer containing concentrations of divalent cations (Mg^{2+}) to produce the controlled expansion of chromosomes with the separation of chromosomal fibers. This approach was extremely successful and the results indicated that a basic mode of fiber organization was as radial loops. It was not the intention of the investigation to resolve fine details of the nucleosomal substructure of the fibers. The use of a relatively thick (8 nm) coating of Au/Pd was therefore more than adequate considering the intent of the experiments. The results were, however, tantalizing in suggesting that SEM is capable of providing a considerably higher resolution picture of chromosome substructure than most electron microscopists apparently believe is possible. Further studies which combine improved SEM techniques with the experimental innovation of expanding chromosomes by adjusting the Mg^{2+} concentration should provide fruitful results. Details of fiber substructure may become clear either by lightly coating chromosomes (perhaps using "cool" sputtering with Au/Pd as suggested by Reviewer III) or by applying the osmium impregnation protocol (text reference 17).

A. Iino: Are there any micrographs which show complete loops in TEMs of thin sections of chromosomes?

Authors: Although transverse sections of chromosomes show a radial arrangement of fibers for samples in 1.0-1.5 mM Mg^{2+}, only rarely can looping fibers be traced continuously from the central region of the chromatid to the periphery and back again. In favorable cases, a number of complete loops can be followed in a transverse section of a particular chromosome. The major unanswered question concerns how these loops are connected. That is, what is the path of the fiber from one loop to another through the central region of the chromatid. Reducing the Mg^{2+} concentration below 1.0 mM expands each chromosome into a tangled network of fibers and it is impossible to follow the path of a fiber for any distance.

A. Iino: What do the authors think about the relationship between decreasing the Mg^{2+} and the clarity of chromatin fibers?

Authors: An advantage of adjusting the magnesium concentration to control the extent of chromosome expansion was that the 30 nm "solenoid" of nucleosomes remained intact. The paper that originally characterized the 30 nm fibers as a superhelix or "solenoid" of nucleosomes utilized solutions containing 0.2 mM Mg^{2+} to stabilize the fibers (text reference 14). This was twice the concentration required

to neutralize phosphate groups under their conditions. Chromosomes resuspended in buffers composed of 5.0, 1.5, 0.5 or 0.15 mM Mg^{2+} would therefore certainly possess the characteristic 30 nm solenoidal fibers. Even concentrations as low as 0.05 mM appear to stabilize these fibers under the solution conditions used for text figures 6 and 7. Discounting the thickness of the metal coating, the fibers in figs. 6 and 7 have a measured width of close to 30 nm. They also have a 15 nm periodicity along the fibers which probably represents helical turns of the 11 nm-wide filament of nucleosomes.

A. Iino: Are the conclusions from your investigation compatible with the SEM observations of chromosomes reported by Utsumi (Utsumi KR (1982) Scanning electron microscopy of Giemsa-stained chromosomes and surface-spread chromosomes. Chromosoma, 86, 683-702)?

Authors: Utsumi reported that minimally stretched chromosomes were composed of nodular and twisted looping fibers having an average diameter of 30 nm. The kinetochore was distinguished as a constriction, and several fibers parallel to the axis of the chromatid could be observed. The organization of the fibers was disordered rather than regular. The observations of Utsumi and the results in this paper are clearly compatible, and both illustrate the complexity of the experimental problem that is being tackled. Looping fibers were detected but their structural arrangement remains a mystery.

T.D. Allen: Do you consider that there is any good evidence for the supersolenoid model currently?

Authors: The proposal of Bak et al (text reference 7) that metaphase chromosomes are composed of a hierarchy of helices that includes a 40 nm "supersolenoid" was based upon observations of partially disintegrated chromosome preparations. Long, tubular structures, that TEM proved to be hollow, were occasionally detected. Bak et al considered these 400 nm tubes to result from the unravelling of the final level of coiling of some chromosomes in the preparations. However, this evidence is unconvincing to many investigators, and the tubes seem likely to be abnormal particles unrelated to native chromosomes. More compelling evidence for a hierarchy of helices comes from light micrographs which show coiled chromatids. But these may arise artifactually from the special treatment applied, which includes acid fixation of the samples. Whole mount electron micrographs rarely display helical regions along chromatids. In addition, the G-banding patterns of extended prophase chromosomes suggest that the transition to metaphase involves contraction of the chromosome length instead of coiling. A possibility remains, however, that the radial loop arrangement is further coiled. The loops, for example, could be arranged along a helix whose axis is coincident with the chromatid axis (text figure 7). Such a symmetric distribution would provide an efficient pathway for the folding of the chromatin fibers during mitotic chromosome condensation.

T.D. Allen: The chromosome shown in fig. 3 (and 2 to a lesser extent) appears to be covered in spherical projections. Whilst this is a possible result of the looped structure of the 30 nm chromatin, they have much more of a "domed" appearance than say the loop of a hairpin. Would the authors like to comment on this?

Authors: An initial concern was that the knobby surface substructure of the chromosomes actually was due to contaminating cytoplasmic particles, such as ribosomes, that adhered to the chromosomes during isolation. TEM of thin sections showed, however, that the surfaces of chromosomes were free of adhering contaminants. Also, the general conclusion from the micrographs of thin sections that the fibers are arranged as radial loops was compatible with the SEM results. The diameters of the surface projections responded to a decrease in the concentration of Mg^{2+} as would be expected for the peripheral tips of chromatin loops. The decrease in the average diameter could be understood as reflecting a loosening or untwisting of highly condensed loops. The minimum average diameter of 30 nm found in low Mg^{2+} was identical to the fiber width. And the number density of the protuberances was reduced as the Mg^{2+} concentration dropped to 0.15 mM and lower. Under these conditions, an increasing proportion of the chromatin was seen as fibers flattened onto the specimen support surface. These considerations argue that the surface protuberances are the structure of peripheral chromatin. The "domed" appearance mentioned by Dr. Allen probably results from the thickness of the Au/Pd coating and the tendency of evaporated metal to accumulate on projecting structures.

Reviewer V: How do your results relate to the SEM investigation of Hanks and colleagues (Hanks et al (1983) Chromosoma, 88, 333-342) and the TEM study of Mullinger and Johnson (Mullinger AM, Johnson RT (1980) Packing DNA into chromosomes. J. Cell Sci., 46, 61-86) which report the presence of longitudinal fibers within the chromatid core surrounded by looping fibers?

Authors: Our results, combining SEM and TEM of intact but slightly expanded chromosomes, are compatible with the conclusions of Hanks et al concerning the arrangement of the 30 nm fiber within prematurely condensed chromosomes at various stages of the cell cycle. They are also compatible with the observations of Mullinger and Johnson dealing with histone-depleted chromosomes. We found that the predominant mode of chromatin fiber organization was a radial array of loops. But the appearance of the micrographs did not rule out the presence of central, longitudinal fibers. Indeed, SEM of some chromosomes in low concentrations (0.15 mM) of magnesium strongly suggested the presence of longitudinal fibers. But chromosomes were readily distorted to differing extents during preparation for EM. To declare that longitudinal fibers are a basic organizational mode would therefore appear to overinterpret our images.