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Chromatin freeze fracture electron microscopy: a comparative study of core particles, chromatin, metaphase chromosomes, and nuclei

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ABSTRACT

Chromatin gels, metaphase chromosomes, and intact nuclei were studied by freeze fracturing followed by electron microscopy. The results complement and extend those obtained by classical electron microscopy techniques as they are obtained without fixation or dehydration. The freeze fracturing technique permits a determination of the hydrated diameters of nucleosomes in chromatin and in nuclei to be 13 nm by comparing to simultaneously studied test objects. Nucleosomes in chromatin fibers are closely spaced but are discrete particles in all conditions studied. In the presence of divalent ions, most chromatin in solution, chromosomes, and nuclei is organized into fibers whose thickness is larger than 40 nm. The images are not at all compatible with a super bead organization of the nucleofilament. Freeze fractures of intact nuclei provides information on the distribution of chromatin in a hydrated unfixed state. The images suggest that most of the chromatin is localized in large domains in contact with the inner nuclear membrane.

INTRODUCTION

Electron microscopy has played a central role in the study of chromatin structure as details from the 2 nm to the micron level can be examined. Early work was directed toward the determination of the number of DNA molecules in the unit nucleoprotein fiber (1,2,3,4) and to an estimation of diameters. More recently the presence of nucleosomes and their distribution with respect to their neighbor and gross morphology have been of most interest (5,6,7). The linear array of nucleosomes or "nucleofilament" (8) was suggested to be first coiled into a loose "quaternary super coil" (9). The external diameter of this loose coil is about 25 nm (10,11,12,13).

The fixation, dehydration, staining, and carbon support-macromolecule interactions required for classical electron microscopy obviously distort the native superstructure and are known to cause large variations in fiber thickness (7,14). Freeze fracturing with subsequent replication

and electron microscopy allows a visualization of unfixed material (see for example (15)). This technique has been extensively and quite successfully applied to the study of membranes (16) where the orientation of this fracture plane is approximately known with respect to the object. Brief studies of nuclei in situ by freeze fracturing have been published (17,18), but as only one micrograph was presented in each case it is difficult to evaluate the results. Our initial freeze fracture studies on chromatin solutions (11,19) showed that this technique could furnish useful information on nucleosome ultrastructure and superstructure.

Here we present new higher resolutions results obtained with a superior appartus and compare the freeze fracture images of chromatin structure over a large range of its possible configurations and associations. We address our attention to the arrangement of nucleosomes in chromatin fibers and are especially interested in the packing and association of these fibers in the presence of divalent ions.

MATERIALS AND METHODS.

Nuclei and chromatin were prepared by the method of Panymin, Bilik and Chalkley (20) omitting Triton-X treatment. The nuclei after pelleting in 2.3 M sucrose were then washed twice in 10^{-5} to 10^{-3} M $CaCl_2$ or $MgCl_2$. For studies of chromatin the nuclei were partially broken by a few strokes in a Potter-Elvehijen homogenizer. A short homogenization with a Pasteur pipet followed. (Phase and electron microscopy implied that about 50% of the nuclei in 10^{-3} M divalent ion were actually broken.) 145 base pair core particles were obtained from chicken erythrocyte nuclei and were a gift from G. Demercia and M. Daune.

Chinese hamster metaphase chromosomes were prepared by nitrogen cavitation according to Wray and Stubblefield (21) in 1.0 M hexylene glycol (Eastman Organic, Rochester, N.Y.), 0.5 mM $CaCl_2$, and 0.1 mM PIPES buffer Calbiochem (San Diego, California)] at pH 6.8. Chromosomes were purified by sedimentation through a 10% to 50% sucrose-buffer gradient in a Sorvall SZ-14 zonal rotor at 2500 x g for 30 minutes (22). Light, electron, and scanning electron micrographs of these preparations displayed the expected configurations and served to monitor their purity.

Freeze fracturing was carried out with two apparatus:

I. with Balsers sample supports and apparatus (301 E) in the usual manner. One microliter drops of suspensions were placed on a platinum support and then plunged into liquid Freon 12 cooled with liquid nitrogen.

Fracturing was with a metal knife cooled to -180° C with the sample at -150° C. Shadowing with 1.5 nm of carbon platinum was carried out in all cases at a nominal angle of about 45° followed by 15 nm of carbon. An electron gun was used in all cases with a 2 x 10^{-6} Torr. vacuum.

II. with the device built by Escaig (23), a vacuum of 1 to 5 x 10^{-8} Torr. Samples were rapidly frozen in liquid nitrogen at -210° C in a sandwich about 20 u thick between two 25 micron thick disks of copper (24). Fracturing was accomplished by separating the two copper disks, and shadowing with platinum-carbon immediately followed. The platinum grain size and contamination of the fractural surface are considerably reduced with this device at 5 x 10^{-8} Torr. (20). With the rapid freezing method (24) non-aggregated particles are observed, without using cryoprotectants. However, with the normal technique (I) 10-20% glycerol was needed to prevent eutectic formation.

After cleaning in the normal manner in sulfuric acid and clorox, the replicas were picked up on unsupported grids and examined in a Seimens 101 or Philips 300 electron microscope. Direct magnifications of 8000 to 40,000 were employed.

Diameter calibrations were carried out by simultaneously freeze fracturing ferritin solutions along side of the frozen chromatin. The shadow widths were measured only on particles or nucleosomes which could be visually distinguished as being separate. Several histograms on solutions of chromatin were made on different micrographs. Each histogram contained 100-200 measurements for chromatin in solutions. Full width half maxima for nucleosomes were 4 nm and 2-3 nm for ferritin. Outer diameters for chromatin were multiplied by the factor: known ferritin diameter/measured ferritin shadow diameter. We thereby obtained the corrected value. Outer diameters for ferritin determined directly from the shadow width, ranged from 11 to 13.5 nm for different freeze fracture preparations. (A detailed account of our freeze fracturing on ferritin will be given elsewhere (LePault, in preparation). Since these values equal the known diameter of 12.2 nm from X-ray scattering (25) within 15%, we feel that our measurements on core particles and chromatin should also be correct.

RESULTS.

Core particles.

Electron microscopy of freeze fractured core particles is of

interest as it can be compared to that in the crystalline form and can serve as a control for other studies on chromatin. Figure 1 shows an electron micrograph of a replica of freeze fractured core particles after rapid freezing. Individual particles of about 10 nm diameter are observed. The diameter was measured by comparison to ferritin molecules freeze fractured simultaneously with and micrographed on the same day as the core particles. The full width half maxima of the shadow widths is almost twice as large for core particles as for spherical ferritin molecules. The average diameter measured from several histograms on different micrographs corrected by reference to ferritin is 9 nm.

The core particles do not seem to be aggregated nor have they been pushed close together with the rapid freezing technique. Still, the possibility of some concentration of particles (eutectic formation) during freezing may not be eliminated. However, these results suggest that much more massive chromatin fibers should be even less affected by the rapid freezing technique.

Holes in the ice whose average dimension are about 9 nm are common in the freeze fracture micrographs of core particles and ferritin (see Figure 1). This would imply that little or no etching or contamination has occurred under the fracturing 5 X 10^{-8} Torr vacuum conditions employed for most of this study. Holes are not observed by us in the Balsers which operates at 10^{-6} Torr. (All figures except numbers 2, 5, and 6 were obtained at 5 X 10^{-8} Torr. in the device described by Escaig, 23).

Chromatin gels in the presence of divalent cations.

After freeze fracturing, chromatin gels in the presence of divalent cations display irregular bundles of loosely coiled nucleofilaments, as in Fig. 2. Nucleosomes can be distinguished in the fibers with an average center to center separation of 10 nm. That is, they appear to be almost contiguous. Comparison to ferritin standards yields a nucleosome diameter about equal to 13 nm. There was no significant change in nucleosome dimensions, between 1 mM mono or divalent salt concentrations. However, the fibers are not individual as in our dilute chromatin solutions in monovalent salt (11,19); rather they appear to be grouped into bundles whose thicknesses are larger than 50 nM (Fig. 2).

Metaphase chromosomes were studied in 1 mM excess $CaCl_2$, and in EDTA solutions where the chelator and DNA mole phosphate concentrations were similar. Although the chromosomes in mM calcium generally appear somewhat more compact, to a first approximation the structure resembles

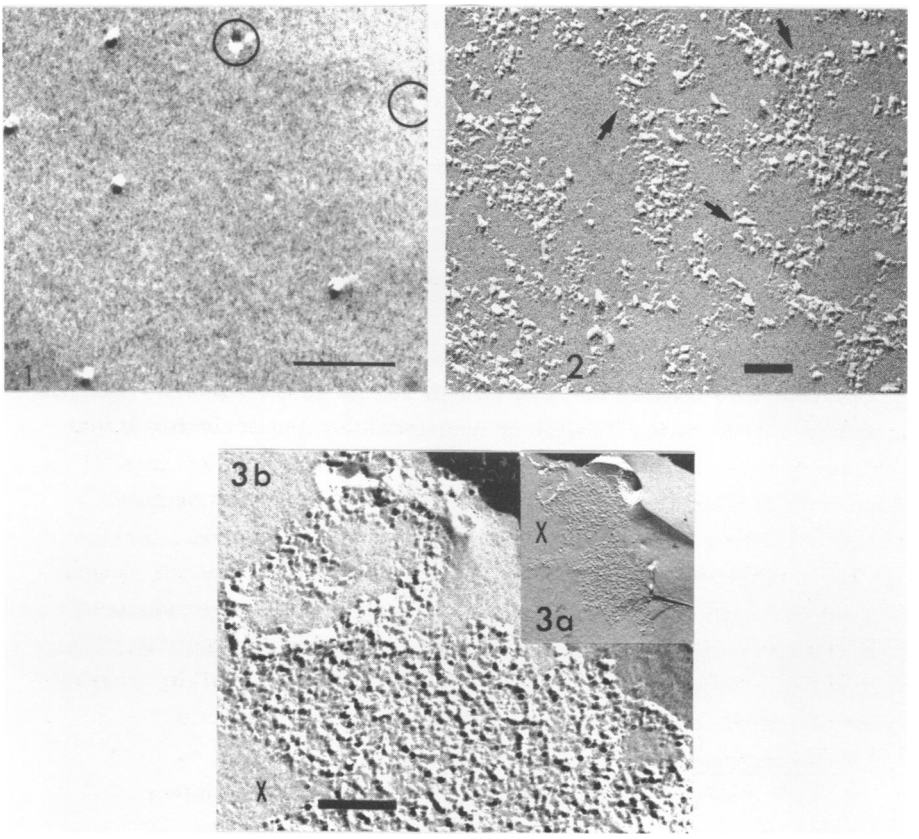

Figure 1. Replica of freeze fractured 145 base pair core particles in 1 mM EDTA, at a concentration of 2 mg/ml. Some cases which appear to be holes are circled. All photographs are presented with the shadowing direction running from bottom to top. Replicated chromatin is black and is associated with white shadows. The bar in all figures represents 100 nm.

Figure 2. Rat liver chromatin fibers (at 2 mg/ml in 20% glycerol) which were always in the presence of 1-2 mM $MgCl_2$. Loose coils of nucleosomes are discerned which seem to pack side by side to form thick fibers 50-100 nm in diameter. The arrows indicate fibers 50 nm in diameter.

Figure 3(a). Freeze fracture of a metaphase chromosome. From the dimensions and aspect it is judged to be a cross fracture of the two chromatids. (b). An enlargement of the region around the (X) of 3a. Note the tightly packed, loosely coiled chains of discrete subunits. Spherical groupings (20-30nm) expected from "Superbeads" (34,35) are not observed.

that when excess free divalent ions have been removed.

Figures 3 and 4 present portions of freeze fractured metaphase chromosomes. The gross morphology and dimensions are similar to chromosomes in the light microsope. Chromatid diameters are 500 to 800 nm. Still, we cannot be certain that we are not visualizing chromatids from more than one chromosome unless and until fractionated metaphase chromosomes may be used in order to compare the conformations in the light microscope with freeze fractured replicas. However, we can make make some general observations on the organizations of chromatin in metaphase chromosomes.

Most of the chromatin in what appear as longitudinal fractures is organized into bundles of fibers 50 to 100 nm wide (Fig. 4). Metaphase chromosome fibers have a subunit or nucleosome aspect in agreement with previous reports (26,27) (Figure 4 C). The ultrastructure of these subunits is similar to that in freeze fractured interphase chromatin (11,19). In the replicas most nucleosomes appear to be almost contiguous with their neighbor. Fifty nm bundles of fibers are the most frequently observed configuations in our images. At the exterior of chromosomes, cross fractures of 50 nm fibers are quite common (as in Figure 4). Twenty-five nm thick fibers are rarely noticed. We have not yet observed a central protein matrix (28) or scaffold (29).

Freeze fractured nuclei.

We have attempted to freeze fracture nuclei under a variety of conditions and so far have obtained satisfactory results with 10^{-5} M $CaCl_2$ or $MgCl_2$ containing 10-20% glycerol (30). Light microscopy shows that nuclei maintain their gross morphology in 10^{-5} M divalent ions, and this is close to the calcium concentration estimated *in vivo* (31). Nuclei frozen in 10-20% glycerol and then freeze fractured appear very similar to those observed *in situ*. In the absence of glycerol we rarely find intact nuclei in our replicas. Instead chromatin like bundles abound. Pellets of nuclei frozen, and then thawed, and observed in the light microscope exhibit the unfrozen morphology in 10-20% glycerol. These are aggregated or broken in only 10^{-5} M divalent ion (30).

Neutron scattering on suspensions of nuclei prepared under similar conditions exhibit a strong maximum between 30 and 40 nm spacings (Baudy and Bram, in preparation). It has been suggested that these large spacings are indicative of the presence and maintenance of some higher order structure (9).

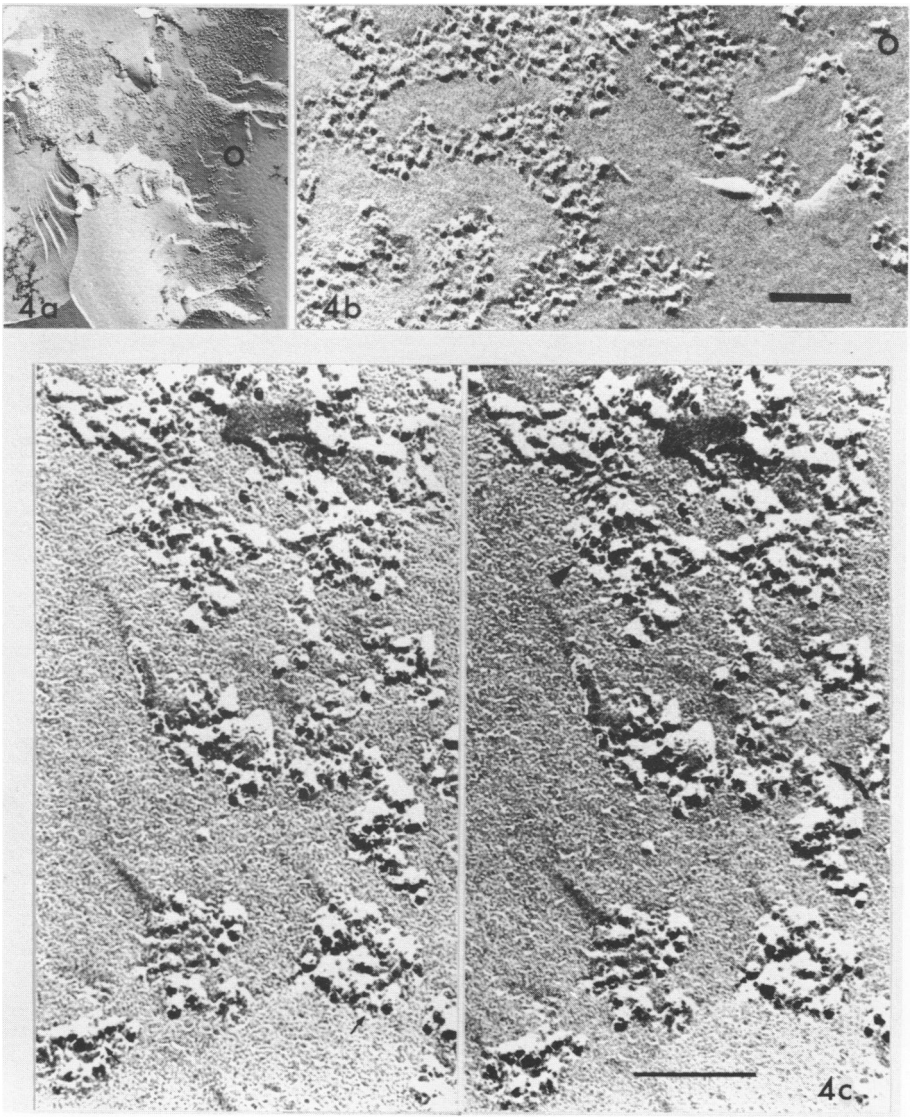

Figure 4(a). A longitudinal fracture of a metaphase chromosome which exposes networks of 50-80 nm fibers. (b). An enlarged view in which the (O) marks the same position in both micrographs. Most material is in 50-80 nm bundles of fibers made up of closely spaced discrete nucleosomes. (c). Stereo views of a longitudinal fracture through the same preparation as a and b. The arrow head shows a stretch of five discrete closely spaced subunits. Small arrows point to what appears to be a 2 nm fiber joining nucleosomes.

The distribution of chromatin in the replicas of fractured hydrated nuclei (Figures 5 and 6) is not uniform. Most chromatin appears to be distributed close to and in contact with the nuclear membrane. Fifty to 200 nm diameter bundles resembling those in our replicas of chromatin containing divalent ions and in metaphase chromosomes are observed (Fig. 6). A 13 nm thick fiber seems to be the smallest structural unit. These unit fibers are seen to be made up of nucleosomes (Figures 5 and 6) and have a similar morphology to that in isolated chromatin and chromosomes.

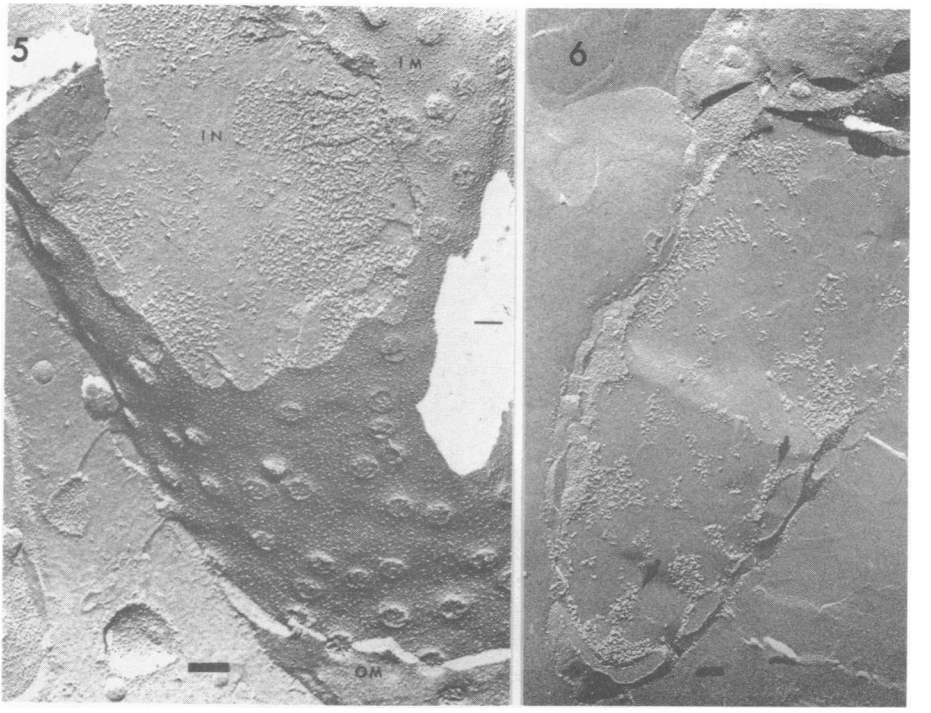

Figure 5. Freeze fractured pellet of rat liver nuclei in 10^{-5} M $CaCl_2$, 20% glycerol. Note the outer nuclear membrane (OM), inner nuclear membrane (IM) and the exposed inside of the nucleus (IN). 90 nm pores and 9 nm particles are associated with the membranes. The domains of fibers made of nucleosomes tend to cluster about the inner periphery of the nuclear membrane. Nucleofilaments of 13 nm diameter nucleosomes are discerned in the nucleoplasm.

Figure 6. A freeze fracture plane through a dilute suspension of nuclei in 20% glycerol. Clusters or domains of chromatin fibers seem to be more prevalent close to and project from the nuclear membrane. Many 50 nm thick fibers (i.e. arrows) are observed.

Nuclear envelopes have particles associated with the membranes "PAM" whose diameter has been measured by several workers to be 9 nm (32,33). Histograms from our micrographs give a value of 8 nm for the PAM particles and 12 nm for nucleosomes from the same micrograph. Assuming that the more extensive membrane freeze fracturing measurements are somewhat more correct, we deduce a corrected nucleosome diameter in nuclei of 14 nm. The 50% larger nucleosome diameter compared to the "PAM", and the observation of the outer then inner nuclear membranes and then nucleoplasm shows that our fractures have indeed exposed the inside of the nucleus.

Consideration of the results and three super structure models.

Our micrographs contain a lot of information which may be difficult to extract by direct visual inspection, consequently it is helpful to compare our results to expectations from models. We have considered how the current models of nucleosome higher order structure compare to our results. The current kinds of models for superstructure are: A) A nucleofilament 13-14 nm thick is loosely coiled into a 25 nm diameter super coil and it associates with other units or winds on itself to form thicker fibers (9,17). B) In the presence of divalent ions the loosely coiled chain of nucleosomes compacts to form a tight super coil about 25 nm in diameter with a pitch of about 11 nm (8). This has been called a solenoid. C) Kiryanov et al., (34) and Renz et al., (35) interpret the higher order structure in chromatin by spherical groupings of nucleosomes into virus-like balls or "super beads" which are strung together by a DNA linker. From visual inspection of our micrographs we can conclude that they do not at all accord with a super bead model. Our chromatin in the presence of divalent ions or in metaphase chromosomes, and in nuclei has a fibrous structure. It is possible, however, that chromatin adopts a super bead structure under environmental conditions other than those studied here.

Super coil models A and B have the same outer diameter (about 25 nm) however in cross or longitudinal fractures they should have rather different aspects. (A schematic representation of models A and B is given in Figure 7.) Images of cross or semi-cross fractures of a solenoid of nucleosome should exhibit a hollow center about 10 nm wide. A solenoid would be expected to behave like 20 nm diameter TMV rods which exhibit hollow centers in freeze fractures (33). Chromatin in our freeze fracture replicas does not display the kind of fractures expected from a tightly

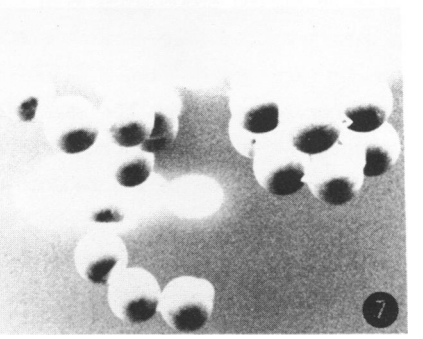

Figure 7. Schematics of two similar current models of nucleosome higher order structure illuminated to mimic the shadowing process used on our micrographs. Shadows are white and are above the black objects. A) Loose quaternary super coil (9), B) Solenoid of Finch and Klug (36). Each sphere is about 12 nm, in diameter, and both coils are 25 nm in diameter.

coiled TMV like solenoid. Instead we observe many individual or double nucleosomes in what appear as in cross fractures and nucleosomes tend to follow the local direction of a loosely coiled 13 nm fibrous unit. Moreover, as mentioned above, 25 nm thick fibers tend to be quite rarely observed in our various replicas of chromatin containing divalent ions, but when they are observed they often seem to be double fibers. Furthermore, they are not at all straight and appear to be super coiled into a 50 nm thick fiber. We feel the best interpretation of our images is that they arise from loose type A model fibers which wind up or double into a 50 nm fiber, and not by a solenoidal model. (Our results would also be compatible with a 250 diameter flat coil or zig-zag of nucleosomes in the absence of divalent ions. Such a model has been drawn to our attention by A. Worcel).

DISCUSSION.

This comparative study of chromatin in solution, in metaphase chromosomes, and in nuclei has given strength to the interpretations in each individual condition. Many features of chromatin in the various states studied here are similar. This is important in itself and allows us to conclude, for example, that these reoccuring features are not artifacts due to isolation or shearing.

It is worthwhile to consider the evidence that we are looking at chromatin in its associations and superstructure(s) and not some artifact. We observe loosely coiled fibers 13 nm thick in all preparations. In solutions the amount of this material visualized is a function of the chromatin concentration and the length observed compares to that expected from biochemistry. In the presence of divalent ions the fibers are highly compacted while smaller dispersed fibrils are seen after nuclease digestion. The local concentration of these fibers is very high in nuclei and metaphase chromosomes but is very low in the remainder of the replica. The general outline of these bundles of chromatin resembles that of chromatids, and in nuclei the exterior surface of the unit fibers is the nuclear membrane.

A discrete subunit structure is observed in our replicas of unfixed hydrated chromatin. This does not accord with a continuous super coiling of DNA along the nucleofilament (36) but suggests that the linker structure differs from that in the central core.

The values obtained for the diameter of nucleosomes by classical electron microscopy vary from 8 to 13.7 nm (6,7,37). Freeze fracturing yields a hydrated diameter on unfixed material, but a simultaneous comparison to a standard is required. Nucleosomes in chromatin solutions, measured with respect to ferritin, have an average diameter of 13 nm. In nuclei direct measurements, by employing internal calibrations on the same micrograph with membrane particles, also yield a 13 nm external diameter. Although this value is 30% larger than generally reported with conventional electron microscopy, it agrees very well with that determined from the neutron scattering on 185 ± 15 base pair nucleosomes (38) or from chromatin fibers (11), and with an indirect linkage model (19,11,39).

In the presence of divalent ions our freeze fracture results suggest that the majority of chromatin is organized into bundles 50-100 nm wide. Photographs of nuclei broken open on electron microscope grids often exhibit such very thick bundles of fibers (7). Prevalent 50-200 nm fibers in nuclei and chromatin containing divalent ions have been reported (40). We find that metaphase chromosomes consist of 50 nm fibers as do Daskal *et al.* (41), and Ris and Korenberg (42) in the presence of .5 to 1 mM Ca^{++}. Twenty nm diameter fibers are rare.

In nuclei, the global distribution of chromatin has been extensively studied by classical electron and light microscopy. Many of these electron micrographs show a localization of 100-200 nm thick chromatin bundles

close to the nuclear membrane (43,44,45). In fact our classical thin sectioning and electron microscopy on rat liver nuclei under similar conditions as used here, are entirely compatible with our freeze fracture images. Light microscopy of whole cells after centrifugation has been taken to suggest a concentration and attachment of chromatin near the nuclear periphery (46,47).

Most of the chromatin in our replicas of freeze fractured nuclei is distributed in domains in contact with the nuclear envelope. The periphery of the nuclei appears to be ringed by a band of chromatin whose thickness is 150 ±100 nm. Rough calculations suggest that all of the chromatin in a rat liver nucleus could be contained in a spherical shell about 5 microns in diameter and 150 nm in width.

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