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Direct imaging in a water layer of human chromosome fibres composed of nucleosomes and their higher-order structures by laser-plasma X-ray contact microscopy

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Summary

X-ray contact microscopy with a 300-ps-duration laser-plasma X-ray source has been used to image hydrated human chromosomes. Clearly imaged are individual nucleosomes and their higher-order particles (superbeads), elementary chromatin fibrils *c.* 30 nm in diameter and their higher-order fibres of various sizes up to *c.* 120 nm in diameter. The results demonstrate that X-ray microscopy is now capable of opening a new path of investigation into the detailed structures of hydrated chromosome fibres in their natural state.

Introduction

Soft X-ray microscopy has many potential advantages over other microscopies for the visualization of thick, hydrated biological specimens in the resolution range 10–100 nm, such as (i) better resolution than optical microscopy and (ii) higher penetration depth than electron microscopy (Kirz & Sayre, 1980). These advantages have led to an increasing effort in X-ray microscope development, particularly in the last few years. Several approaches to high-resolution imaging are now being tried, and have already demonstrated the potential of this new microscopy in visualizing different biological structures. These include the observation of proteoglycan (Panessa *et al.*, 1981), myosin filaments (Panessa-Warren, 1984), living platelets (Feder *et al.*, 1985), sea-urchin sperm (Tomie *et al.*, 1991) and human chromosomes (Shinohara *et al.*, 1992a) by contact microscopy, midge polytene chromosomes by imaging zone plate microscopy (Guttmann *et al.*, 1992), and bean chromosomes by scanning zone plate microscopy (Williams *et al.*, 1992). The latter two methods have not demonstrated sufficient resolution yet to reveal the fine structures of chromatin fibres, including nucleosomes.

Observing fine structures of chromosome and chromatin as intact as possible in a hydrated state is important for better understanding their organization and functions *in situ*. These functions may play a key role in various cellular control mechanisms such as cell cycle progress, differentiation, radiation sensitivities and cell death.

Eukaryotic chromosomes are composed of chromatin fibres which themselves consist of unit structures (Watanabe *et al.*, 1990), nucleosomes of 10–15 nm diameter (Langmore & Wooley, 1975; Oudet *et al.*, 1975) and superbeads of 15–50 nm diameter (Hozier *et al.*, 1977; Azorin *et al.*, 1982; Zentgraf & Franke, 1984). Bordas *et al.* (1986) have shown that the native uncondensed chromatin fibre in solution has an outer diameter of *c.* 30 nm using synchrotron radiation scattering analysis. Belmont *et al.* (1987) have demonstrated using analyses of three-dimensionally reconstructed electron microscopic images of isolated *Drosophila melanogaster* chromosomes that there is a size hierarchy of discrete chromatin structural domains with cross-sectioned diameters of 120, 240, 400–500 and

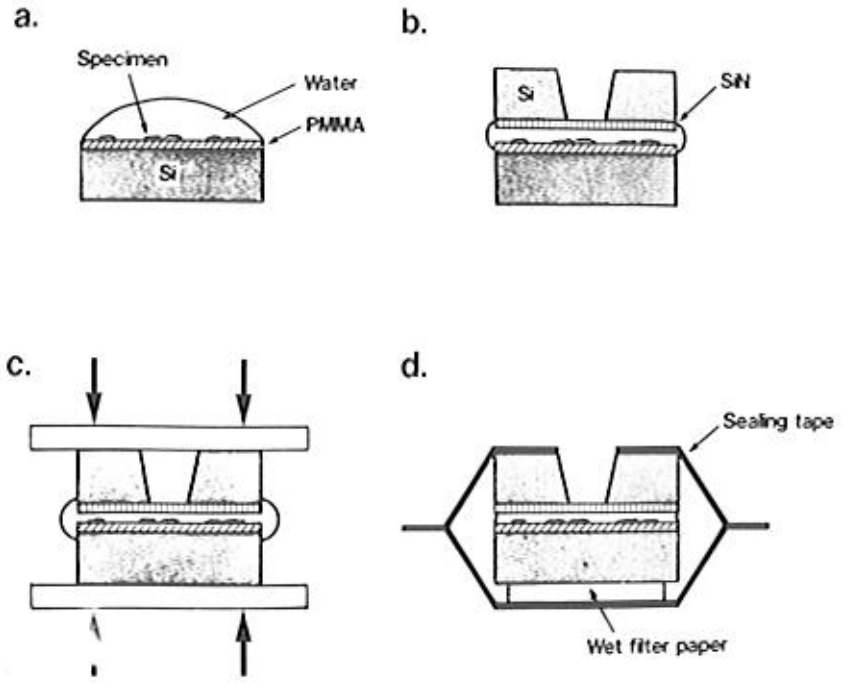


Fig. 1. Experimental assembly of a simple hydrated chamber. The wet specimens isolated on a surface of distilled water were mounted on a layer of PMMA supported on a silicon wafer (a). The wet specimens were then covered with an SiN window (b) and press-fitted with a torque gauge (c). This wet assembly was then sealed with two pieces of adhesive tape, together with a piece of a wetted filter paper (d).

800–1000 nm in mitotic chromosomes. Nevertheless, the organization and functions of chromosomes or chromatins *in situ* have not been clarified yet.

In a previous publication (Shinohara *et al.*, 1990), we have described the first use of X-ray contact microscopy to observe the 'beads-on-a-string' structures in human chromosome fibres. The chromosome fibres were as close to their natural state as possible. Namely, they were unstained without any fixative. However, they were dried, and the drying process may have modified the fine structure of the chromosome fibres. This possible limitation is overcome in the study of hydrated specimens.

For the observation of hydrated biological specimens at high resolution, the exposure time should be less than 1 ms to eliminate the image blurring caused by thermal diffusion and/or radiation damage (Shinohara & Ito, 1991; Ito & Shinohara, 1992). From this point of view a laser-plasma X-ray source (Rosser *et al.*, 1985) is the most suitable for X-ray microscopy at the present time.

Laser-plasma X-ray sources have previously been used for a number of contact microscopy studies of biological specimens (Tomie *et al.*, 1991; Ford *et al.*, 1992; Cheng *et al.*, 1992), including our previous studies (Shinohara *et al.*, 1992a,b, 1994), in which natural hydrated human chromosome fibres were imaged with soft X-ray contact microscopy illuminated by a pulsed (300-ps), single burst of X-rays from a laser-plasma X-ray source. In the present work, we have precisely analysed the results obtained by the X-ray contact microscopy of human chromosome fibres in a water layer with laser-produced plasma X-rays.

Materials and methods

Chromosome preparation in a hydrated specimen chamber

The chromosome specimens were prepared for X-ray imaging as follows. Human lymphocytes (RPMI 1788) were incubated at 310 K (37°C) in RPMI 1640 medium supplemented with 10% fetal bovine serum in the presence of 0·05 μg/ml colcemid for 16 h to accumulate mitotic cells. After centrifugation the cell pellet was placed on a clean surface of distilled water. At this moment, the cells were broken, with the consequence that the chromosome rapidly flowed out across a large surface area of the water. The chromosomes were then concentrated in density on the surface of the water by decreasing the surface area over which they could float. Finally, they were whole-mounted directly onto a thin layer of photoresist, polymethylmethacrylate (PMMA), supported on a 0·4-mm-thick silicon wafer. The thickness of PMMA was 0·7 μm and the overall substrate size was about 7 mm × 7 mm. It should be noted that chromosome fibres attached partially to the surface of PMMA and the rest of them were floating in the water layer.

The hydrated specimens without fixation and staining were then placed in a simple hydrated specimen chamber (Shinohara *et al.*, 1992b). This is shown schematically in Fig. 1. This specimen chamber was assembled in the following way. After the hydrated chromosomes were mounted on the PMMA resist (Fig. 1a), they were immediately covered with a thin (400-nm-thick) SiN square (250 μm × 250 μm) window, ensuring that they were always maintained in a hydrated condition (Fig. 1b).

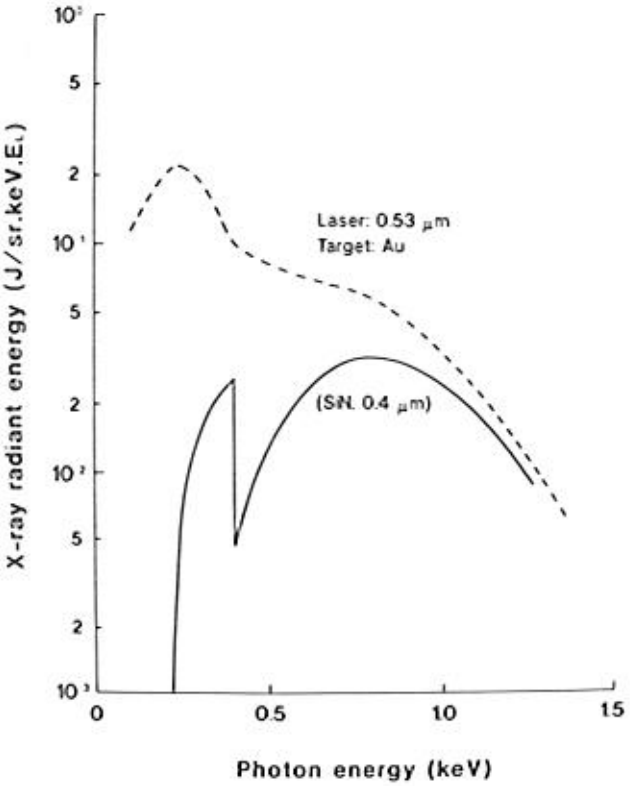


Fig. 2. Spectrum of the X-ray flux emitted from the laser-produced plasma from Au target. The dotted line shows the X-ray flux transmitted through the SiN window.

The small SiN window had been chemically etched into a 0·4-mm-thick silicon wafer having approximate dimensions of 7 mm × 7 mm. While still in a wet state, these two wafers were then press-fitted together using a torque gauge (11·8 N · cm) attached to a micrometer (Fig. 1c). Thus, the chromosomes were encapsulated in a fully hydrated state. The excess water was removed with a piece of a filter paper, and the system was sealed with two pieces of adhesive tape (Scotch tape #483) as shown in Fig. 1(d). A small piece of wet filter paper was included on the rear of the specimen holder in order to verify that the specimen was still hydrated at the time of X-ray exposure. The final thickness of each water cell was in the 1–5 μm range, measured with an optical microscope. Specimen chambers constructed in this manner were found to be water-tight for many hours in a vacuum chamber.

X-ray exposure

The encapsulated human chromosomes were exposed to a single burst of X-rays from a laser-produced plasma. The plasma was created from solid Au targets by the focused second harmonic radiation (527 nm) converted from one beam of the four-beam GEKKO IV Nd:glass laser system at the Institute of Laser Engineering at Osaka University. The 300-ps (FWHM) duration Gaussian output pulse of energy 26 J was focused in an evacuated target chamber (10^{-4} Torr) with a spot size of 100 μm diameter on the Au target, providing an irradiating intensity of *c.* 10^{15} W/cm^2. The X-ray emission from Au targets under these irradiation conditions has been well characterized (Kodama *et al.*, 1986). The specimen cell was situated 2 cm from the target at an angle of 25° to the laser axis and the target normal. Figure 2 shows the spectrum of X-ray flux from the plasma emitted in this direction and the spectrum of the X-rays irradiating the chromosomes taking account of the absorption of the SiN window (Henke *et al.*, 1982).

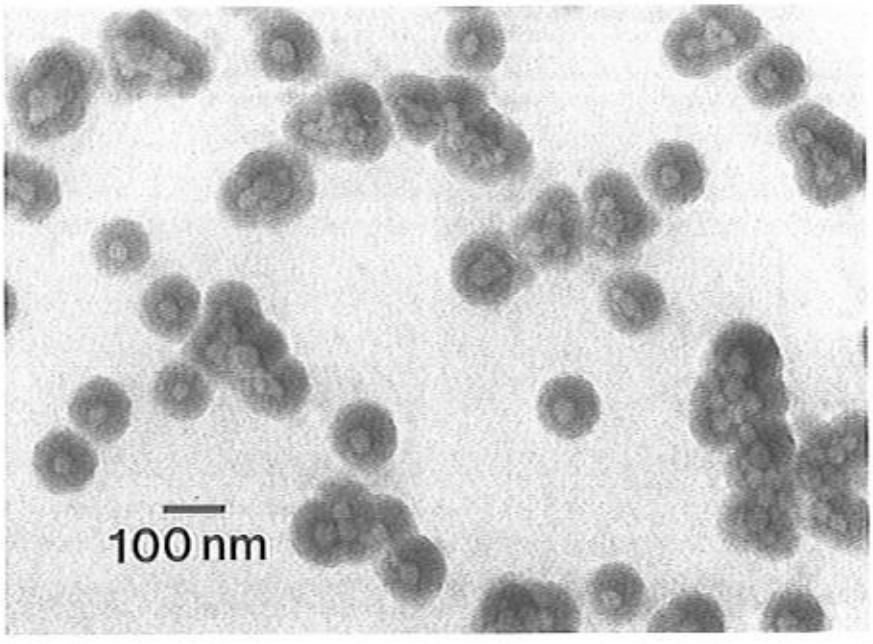
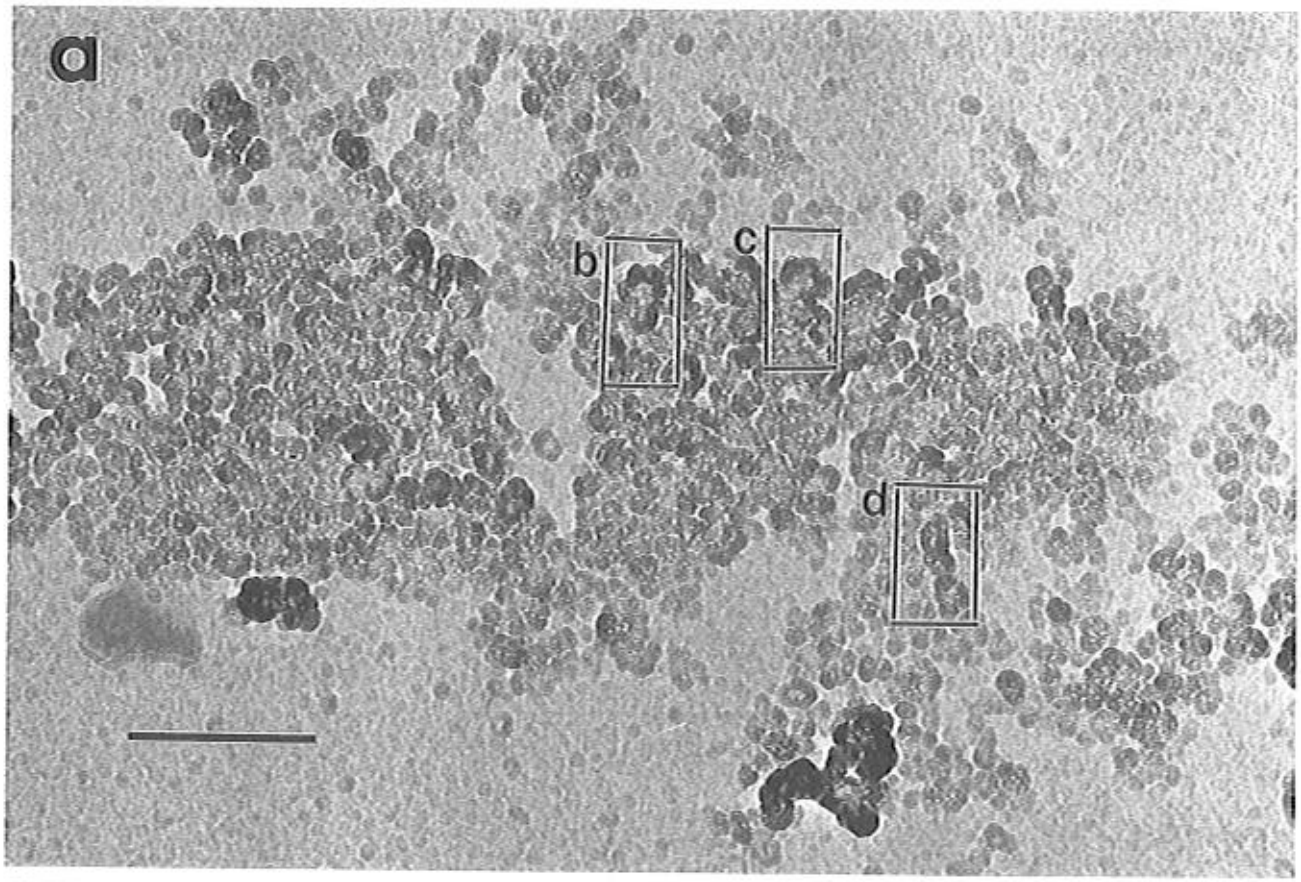


Fig. 3. A replica image of latex spheres. Note that the images of spheres and their aggregates are surrounded by large dark regions.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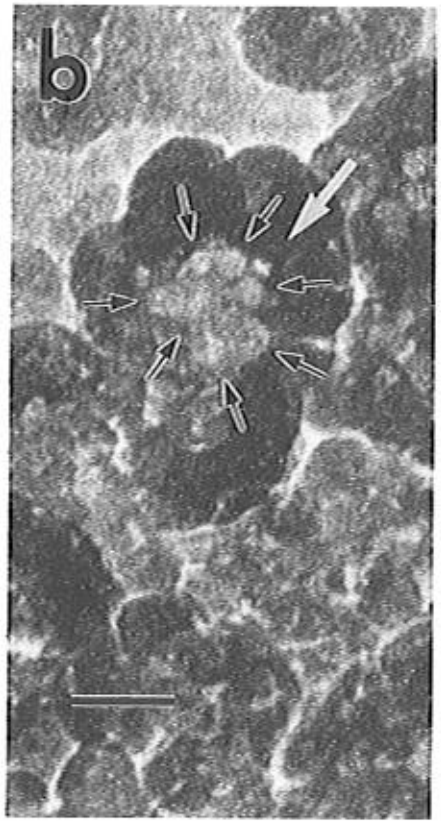
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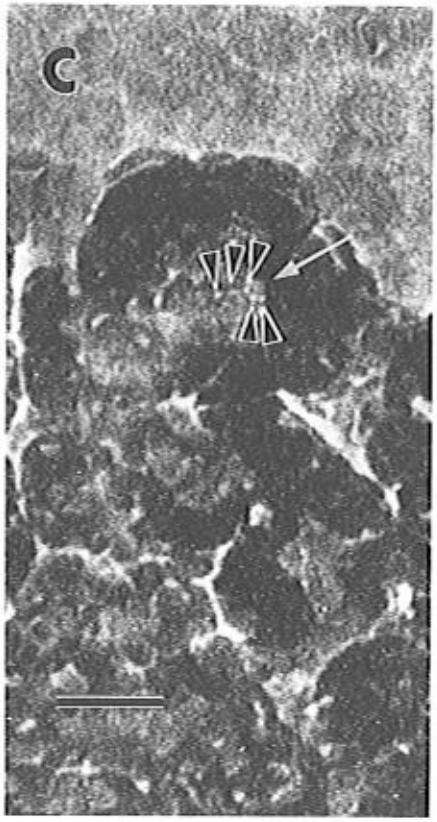
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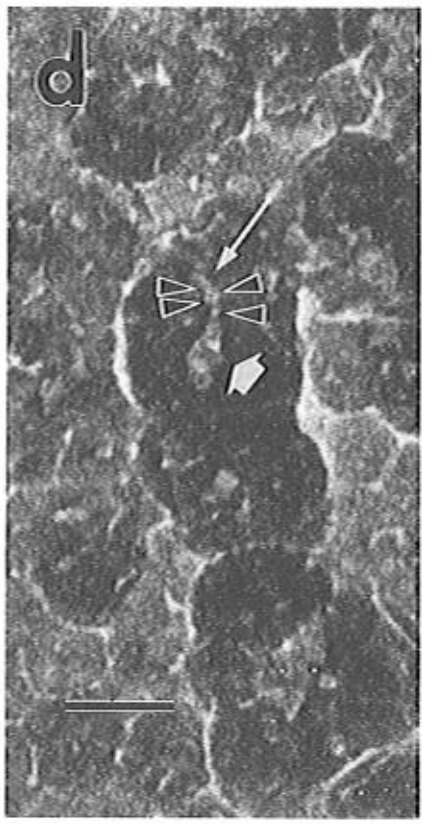
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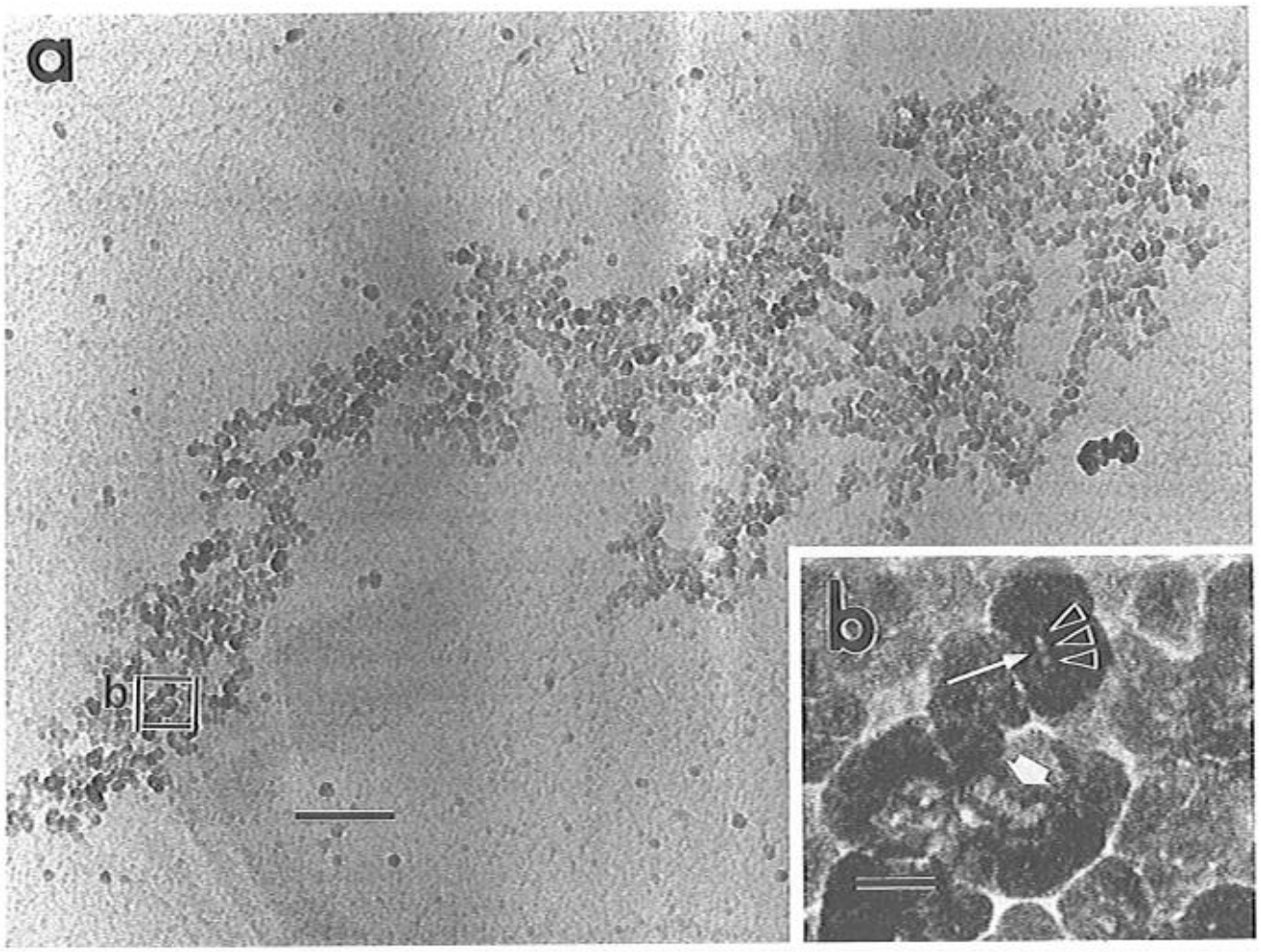


Fig. 5. Another example of a condensed chromosome fibre (a) and an enlarged image of a framed area (b): polynucleosome filaments (thin white arrow) and a 30-nm fibre (short and thick white arrow) with tightly packed nucleosomes are evident. Scale bars = 1 μm (a) and 100 nm (b).

The total photon flux irradiating the specimen was estimated to be *c.* $1{\cdot}4 \times 10^{15}$ photons/cm^2 in the photon energy range of 250–1250 eV. The relative contribution of X-rays at the wavelength range above 4·37 nm, 4·37–2·33 nm (water window) and below 2·33 nm for imaging a nucleosome is estimated to be 1.1, 82·0 and 16·9%, respectively (Shinohara *et al.*, 1992a,b).

Development and observation

After exposure to the laser-plasma X-rays, the specimen chamber was removed from the target chamber and was disassembled. The PMMA was then developed and treated in a procedure previously described (Shinohara *et al.*, 1986). Chromosomes were removed from the PMMA with sodium hypochlorite (chlorine concentration, 0·5%). The PMMA was then developed with a mixture of methylisobutylketone and isopropanol. The three-dimensional topological representation in the PMMA of the absorption of the hydrated chromosome structures was then observed through using the so-called 'replica method' (Tanaka, 1983; Karasaki & Tanaka, 1984) and a transmission electron microscope. The resolution of the replica method is higher than 2 nm (Tanaka, 1983). It has

Fig. 4. An X-ray image of a condensed chromosome fibre (a) and enlarged images of three specific regions identified by framed areas (b–d): (b) a cluster of superbeads about 30 nm in diameter (white arrow); (c) a polynucleosome filament in a series of nucleosomes (thin white arrow); (d) a 30-nm fibre constructed with a polynucleosome filament. Scale bars = 1 μm (a) and 100 nm (b–d).

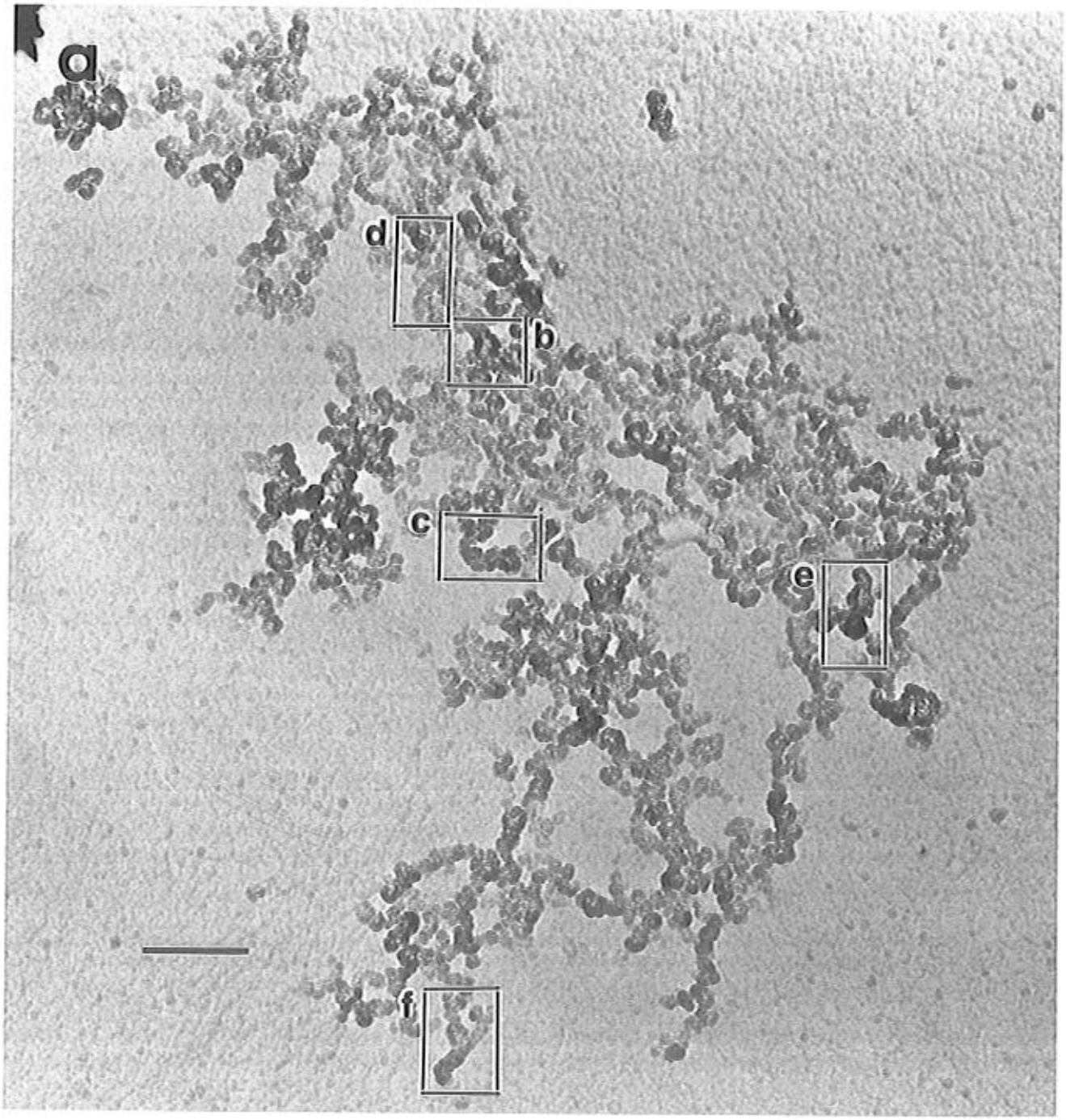


Fig. 6. A partially decondensed chromosome fibre (a) and enlarged images of the framed areas (b–f): (b) a higher-order fibre (thick white arrow) 60–100 nm in diameter constructed with a helically coiling fibre; (c) a knobby 30-nm fibre (small and thick white arrow) comprising superbeads; (d) a polynucleosome filament (thin white arrow); (e) a 30-nm fibre with two regularly arranged rows of nucleosomes (white arrow); (f) a series of nucleosome and superbeads. Scale bars = 1 μm (a) and 100 nm (b–f).

been demonstrated that PMMA has an estimated resolution of up to 5 nm (Gudat, 1982) and is able to identify a single nucleosome (Shinohara *et al.*, 1990). As was discussed in the previous paper (Shinohara *et al.*, 1990), the image observed in a transmission electron microscope represents the white area covered with a dark region. This was explained using a simple model. However, images may be complicated when specimens have structures that extend three dimensionally, such as is the case for the present experiments. In such a case, X-rays pass through

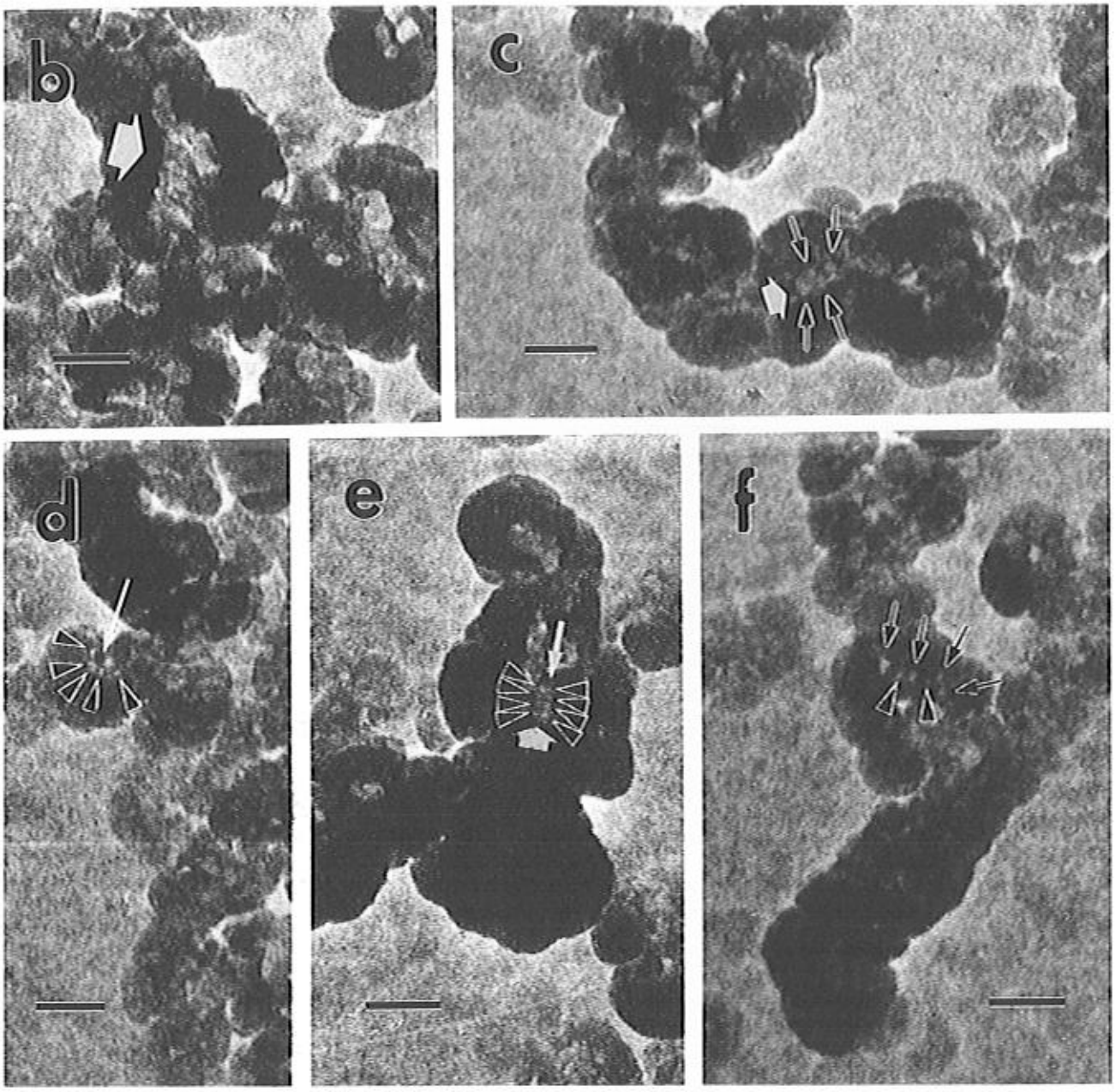
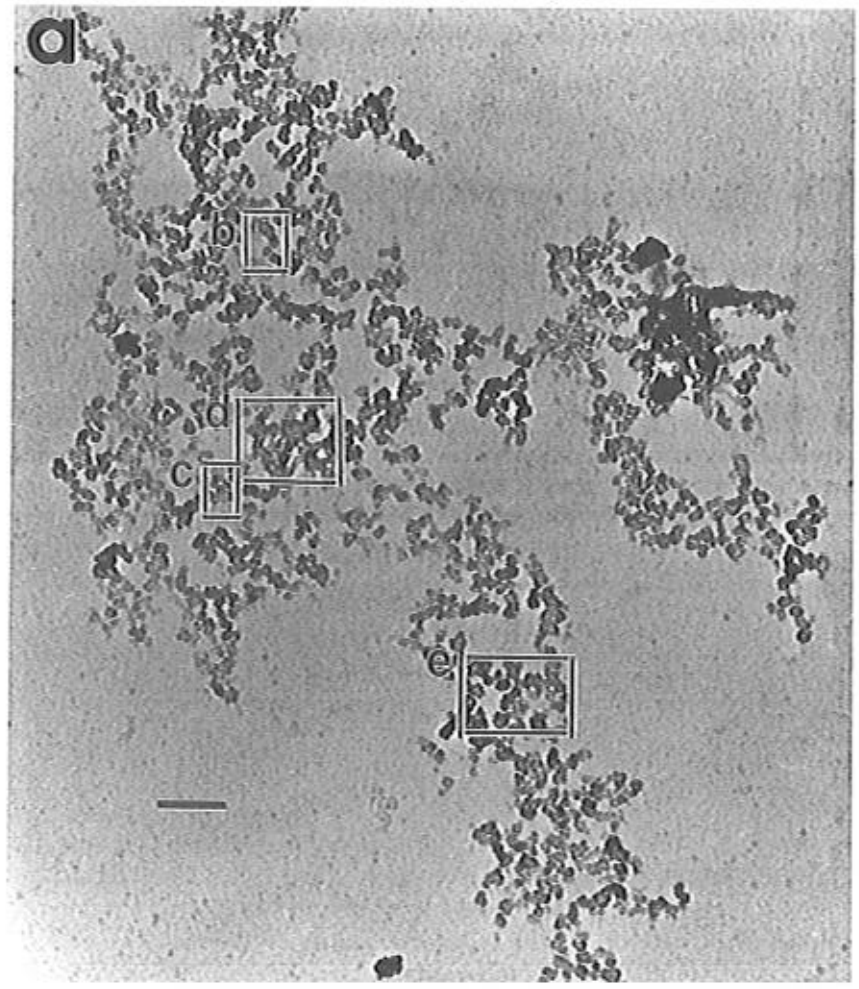
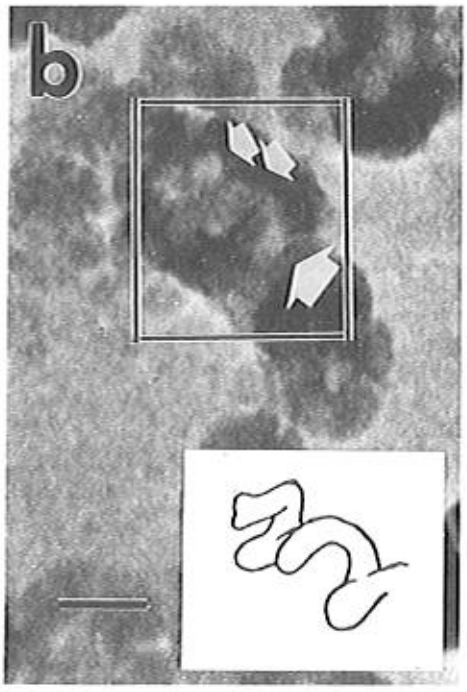


Fig. 6. *continued.*

three-dimensional specimens and reach PMMA. The flux distribution on the PMMA depends on structures and the distance of the specimens to the PMMA. If some structures stay distant from the PMMA, the images projected on the PMMA may be distorted by penumbral blurring and Fresnel diffraction. The amount of distortion depends on the distance. Sometimes they will produce no structures but affect the height of the relief of PMMA after development. Therefore, the relief on PMMA after development will have very complex structures. To understand how such complex structures are observed by the replica method, we tested latex spheres (mean diameter: *c.* 38 nm) and their aggregates as model systems. Latex spheres (Dow Chemical Company) suspended in water were placed onto the surface of PMMA. After evaporating the water a replica film was made by the same method as that used in the present experiment. The remaining latex spheres attached to the replica film were removed by treating the film with toluene before transferring the film to an EM specimen grid. Figure 3 shows the replica image. Their structures were observed as grey areas with dark surroundings. This image coincided with the image expected for a simple model and showed that





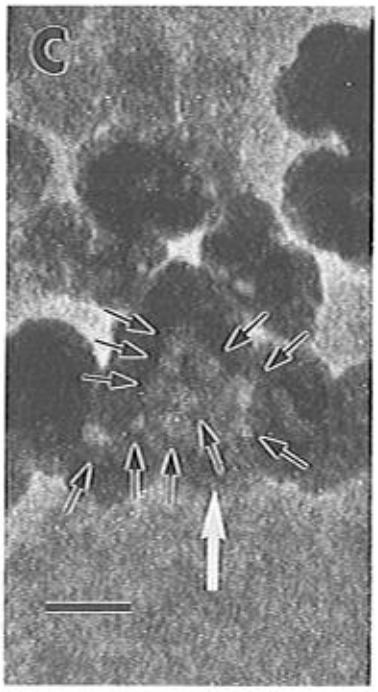


Fig. 7. Another example of a decondensed chromosome fibre (a) and the enlarged images of the framed areas (b–e): (b) a higher-order fibre (large white arrow) with a helically coiling 30-nm fibre (small white arrow); (c) a cluster of superbeads (white arrow); (d) a 30-nm fibre with tightly packed superbeads (small white arrow) and a higher-order fibre 60–120 nm in diameter (large and thick white arrow) comprising a coiling 30-nm fibre; (e) superbeads in a moruloid organization. Scale bars = 1 μm (a) and 100 nm (b–e).

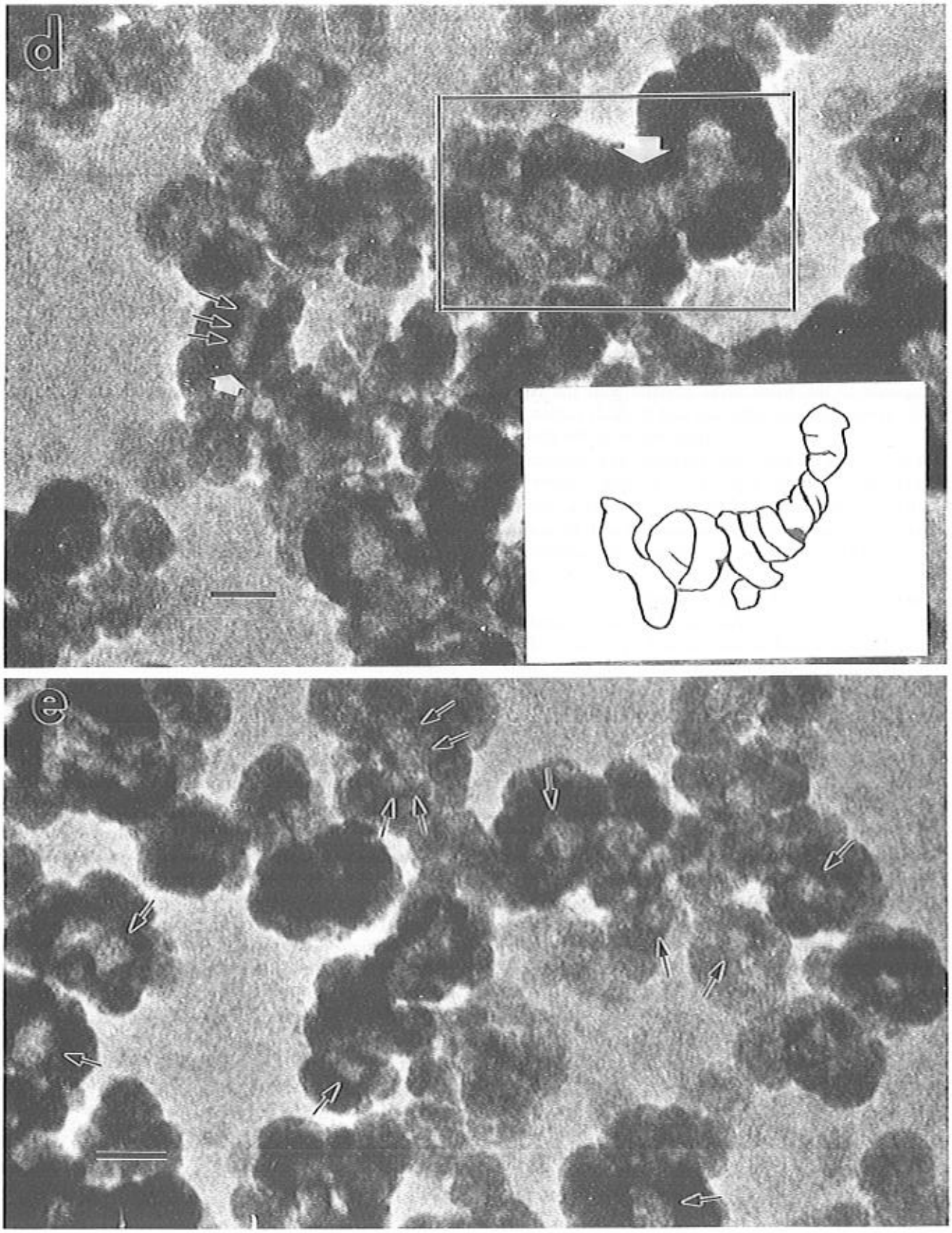


Fig. 7. *continued.*

the model could, in principle, be extended to complex structures. Therefore, the images by the replica method correspond to a white or grey area surrounded by a dark region.

In the present experiments, the observation and analysis were made selectively for the areas covered with dark regions. It should be noted that the images were identified from the background structures by the presence of dark areas at high contrast.

Results

Figures 4 and 5 show X-ray images of condensed chromosome fibres, and Figs. 6 and 7 are images of partially decondensed chromosome fibres. The specimens were always in a hydrated condition during the exposure. In the replica method, the X-ray image from the TEM corresponds to the white areas covered with the black regions as shown in the Materials and Methods section. In Figs. 4–7, (b) to (f) show enlarged images of the framed areas (a). Usually the fibres were entangled in a complicated manner. However, in Figs. 4–7 a wide variety of hierarchical organization of fibres and particles can be observed: nucleosomes (defined as particles with their sizes less than 15 nm in diameter; arrowheads), superbeads (supranucleosomal particles: defined as particles larger than 15 nm in diameter; black arrows), superbead clusters (white arrows), polynucleosome filaments (thin white arrows), '30-nm' fibres (thick white arrows), and higher-order fibres up to 100 nm or more in diameter (large and thick white arrows). In images of the higher-order structures, nucleosomes are often identified as their components. In Figs. 4(b) and 7(c, e), superbeads and their clusters are apparently composed of multiple nucleosomes in a 'moruloid organization' (Zentgraf & Franke, 1984). The mean diameters of nucleosomes (9–15 nm; indicated by arrowheads) and superbeads (16–71 nm; indicated by black arrows) were estimated to be $12{\cdot}6 \pm 1{\cdot}8$ ($n = 27$) and $33{\cdot}8 \pm 9{\cdot}0$ nm ($n = 40$), respectively. Series of nucleosomes are considered to be polynucleosome filaments (Figs. 4c, 5b and 6d), though internucleosomal linker DNA (2 nm in diameter) is not identified because of the resolution limit of the technique. Fibres 30 nm thick are seen in Figs. 4(d), 5(b), 6(c) and 7(b, d) where nucleosome- and/or superbead-sized particles can be seen. In one case (Fig. 6e), the fibre is constructed with regularly arranged parallel rows of nucleosomes (thinner white arrow). Higher-order fibres constructed from 30-nm fibres are seen in Figs. 6(b) and 7(b,d). In Fig. 7(b, d), fibres with diameters of $\geqslant 30$ nm coil helically into thicker (60–120 nm in diameter) fibres (framed by rectangles and the illustrations of those fibres are shown in the column in each figure). Another example of a helical coil is observed in Fig. 6(b). In this case, however, the fibre coils so tightly that it is hard to estimate the internal structures.

Discussion

Specimens in a water layer must stay in partial contact with PMMA, close to it, and distant from the PMMA. The difference in the distance will cause the difference in the images: clear images for the specimen on PMMA, becoming less clear by penumbral blurring and Fresnel diffraction as the distance increases. Therefore, it is not easy to find an image of the long fibrous region of chromosome fibres. Rather, most of the images must be discrete. So far, we have not succeeded in imaging the long fibrous region of chromosome fibres, especially in the images of hydrated specimens. Nevertheless, we believe that the present results are the images for parts of chromosome fibres for the following reasons. (i) The images were basically comparable in their sizes and shapes with those we had observed either by conventional electron microscopy using uranyl-stained specimens (Watanabe *et al.*, 1990) or by X-ray contact microscopy for dried unstained specimens (Shinohara *et al.*, 1990) prepared by the same technique as the present experiments. (ii) We obtained the images shown in Fig. 3 for latex spheres and their complexes as model systems, where the simple model for replica formation was proved also to be applicable to complicated structures such as hydrated chromosome fibres. (iii) Images were identified as those covered with dark regions from the structures in a bright area where there may be images of nucleosomes in weak contrast. Most parts of the bright areas showed a weak contrast of uniform granular structures. Such structure with a weak contrast may be produced by other component(s) of cellular materials, as the same sort of images were observed for dried specimens prepared by the same method either by X-ray contact microscopy (Shinohara *et al.*, 1990) or by electron microscopy (unpublished observation). In the present study, we have succeeded in observing fine structures of human chromosome fibres in a hydrated condition by X-ray contact microscopy with the aid of laser-produced plasma X-rays. The data revealed a wide variety of hierarchical organization of chromosome fibres, which is fundamentally compatible with previous data (Langmore & Wooley, 1975; Oudet *et al.*, 1975; Hozier *et al.*, 1977; Azorin *et al.*, 1982; Zentgraf & Franke, 1984; Bordas *et al.*, 1986; Belmont *et al.*, 1987; Shinohara *et al.*, 1990). One of those examples is 'moruloid' organization of superbeads with nucleosomes, as was reported by Zentgraf & Franke (1984) using conventional electron microscopy with fixation and staining. The present study presents the first evidence of such structures in unfixed, unstained and hydrated chromosome fibres. Since the chromosome fibres were spread over the surface of distilled water, there still remains some uncertainty as to the relationship between the present observation of the hierarchical organization of chromosome fibres and those of *in situ* natural structures for the following reasons. (i) The surface tension may stretch

some of the chromosomes from their natural state. (ii) The ionic environment during the preparation may modify the extent of packing tightness of nucleosomes into superbeads from their natural state.

Nevertheless, it should be emphasized that the current technique is capable of observing a wide variety of hierarchical organizations of chromosome fibres, neither fixed nor stained in a hydrated condition, with a highest resolution of 10 nm. It has been discussed in detail elsewhere (Shinohara *et al.*, 1994) that X-ray contact microscopy can be used to image hydrated biological specimens at a resolution of 10 nm, circumventing the possible causes of artefacts such as radiation damage, thermal diffusion, thermal expansion, temperature increase and the effect of free radicals produced in water.

In summary, we have shown that the direct imaging of a wide variety of hierarchical organizations in hydrated chromosome fibres is now possible with X-ray microscopy with a resolution of up to 10 nm. This introduces a new form of high-resolution imaging to biology. By accumulating data from images of condensed and decondensed chromatin fibres *in situ* in their natural state, much useful information can be obtained on the organization and on the regulation of the function of chromosomes. In addition, in the future, the use of coherent X-rays from pulsed X-ray lasers and X-ray holographic techniques will provide three-dimensional images, from which detailed information on the higher configuration of nucleosomes can be obtained.

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