THIN FILMS OF INTERPHASE CHROMATIN PREPARED FOR ELECTRON MICROSCOPY BY OSMOTIC SHOCK TECHNIQUE

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ABSTRACT. — Interphase chromatin of chicken erythrocytes does not spread in a langmuir trough. By a new technique of osmotic disruption it spreads into a mass of knobby 70-625 Å wide fibers which aggregate to form dense chromocenters. The new method is simpler and faster than the conventional one. Enzymatic digestions indicate that DNA is necessary for the linear continuity of nucleoprotein fibers and histone seems to be necessary for fiber aggregation and the condensation of chromatin.

With the advent of the electron microscope, it was anticipated that the internal organization of hereditary material in higher organisms could be resolved. But except for the synaptinemal complexes in paired meiotic chromosomes (Moses, 1960), examination of thin sections has offered very little information. Thus, the recent discovery by Gall (1963) of a method for spreading nuclear content into monolayer films in a ''Langmuir trough'' represents a technical breakthrough. Gall's technique has been used successfully by several investigators to study the fine structure of interphase chromatin, and mitotic and meiotic chromosomes in quite a few different organisms (Ris and Chandler, 1963; Gall, 1966; Wolfe, 1965a, Wolfe, 1965b; Wolfe and John, 1965; DuPraw, 1965). However, nuclei of certain cell types do not spread readily in a Langmuir trough. For example, chicken nucleated erythrocytes and human sperm heads spread very little or not at all while those of amphibian erythrocytes and grasshopper sperms spread well (Fig. 1a, b). Apparently, the former possess a rather rigid membrane system. Removing the membranes of human or bull sperm heads by alkaline thioglycolate exposes their nuclei and gives adequate chromatin spreading (Lung, 1968).

Chicken erythrocyte nuclei can be spread by osmotic shock (Fig. 1b, c). This method is a modification of the protein monolayer technique devised recently by Freifelder and Kleinschmidt (1965) to spread isolated viral DNA.

MATERIALS AND METHODS

One ml of rooster's venous blood was collected in a heparinized syringe and washed 3 times in 0.01 M Tris buffer containing 0.003 M $MgCl_2$, pH 7.5. Red blood cells were sedimented at 200 g for 5 min. They were then resuspended in hypotonic solution or distilled water and centrifuged at 2000 g for 10 min. This step was repeated 2 or 3 times until an upper layer of intact free nuclei was obtained.

The free nuclei were suspended to a final concentration of about 10^5/ml in a hyperphase solution of

1.0 M ammonium acetate, 0.5% neutralized formaldehyde and 0.01% cytochrome c (Nutritional Biochem. Corp.). A volume of 0.01 ml of well mixed hyperphase was poured down an inclined wet glass slide into a petri dish containing chilled hypophase solution of 0.3 M ammonium acetate and 0.5% neutralized formaldehyde. Excellent results in delivering such a small volume was obtained by using a 10 lambda Ependorf micropipette (Brinkmann Instruments).

The surface film was picked up by touching it with a Formvar-carbon coated copper grid. Immediately after picking up the surface film, uranyl staining was achieved by dipping the grids in freshly pre-

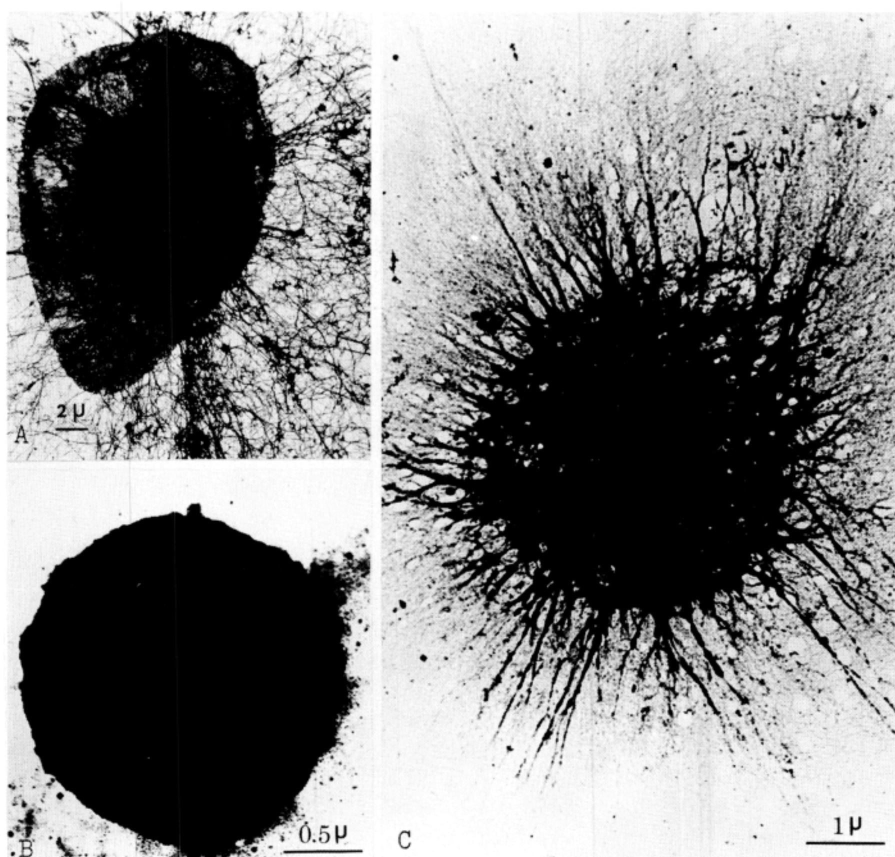

Figure 1. (a) A single erythrocyte nucleus of the Mexican salamander *Ambystoma mexicanum* spread in a Langmuir trough into essentially a monolayer preparation. It shows the dense fibrillar components of interphase chromatin in these cells. X 2,090; (b) The erythrocyte nucleus of a chicken remained intact after being spread in a "Langmuir trough". X 2,010; (C)A single erythrocyte nucleus of chicken spread by osmotic shock technique into a mass of knobby chromatin fibers. X 10,400.

pared Wetmur's ethanol solution of the stain (Wetmur et al., 1966) for 30 sec. and then dehydrated for 15 sec. in chilled isopentane followed by air drying.

In DNase, RNase or trypsin treatments, digestion was carried on prior to staining. Trypsin (Sigma, 2X crystallized) and RNase (Nutritional Biochem. Corp. Crystalline) solutions were prepared as 1.0 mg/ml and 0.1 mg/ml of glass distilled water respectively. But DNase (Worthington deoxyribonuclease I, electrophor. purified) was prepared as 0.1 mg/ml of .003 M $MgCl_2$ and .01 M Tris buffer, pH 7.5. Stock solutions of the hyperphase, hypophase, Wetmur's stain, and Tris buffer were all millipore filtered at least once. The grids were examined in a JEM-T7 at 60 KV and an EMU-3 RCA at 50 KV.

Results

Interphase chromatin of chicken erythrocytes spreads into an interconnected mass of 70-625 Å wide nucleoprotein fibers. Except for the 70 Å fibers which seem to be the basic structural unit of chicken chromosomes, two parallel or relationally coiled sub-units were often observed within the wider fibers. At first glance the 625 Å fibers seemed to consist of two 300 Å units, the 300 Å fiber of two 150 Å units and the latter of two 70 Å units. However, fibers show definite polarity in their diameter tapering off from their base outward. They also exhibit a random pattern of branching into, or association between, fibers of different widths. Thus, observed differences in fiber diameter could not be explained by simple association in twos of equal size subunits. Chromatin fibers are spaced irregularly with chromomere like knobs and aggregate into a thick mass of chromatin similar to chromocenters.

Digestion with 0.1 mg/ml DNase for 15 min. disrupted drastically the linear continuity of chromatin fibers leaving a heterogeneous mixture of granules and ghost fibers (Fig. 2a). Aggregates of electron dense granules represent the remnants of condensed heterochromatin. Treatment with 0.1 mg/ml RNase for 15 min. showed no marked change in chromatin fiber diameter or continuity (Fig. 2b). Both knobby appearance and aggregation of fibers into a chromocenter were still evident. Digestion with 1.0 mg/ml trypsin for 30 min. resulted in dissociation of thick fibers into thinner ones and a reduction in their diameters to 40-100 Å with no loss in fiber continuity (Fig. 2c). Here, however, the fibers were smooth, largely free of knobs and the chromocenter appeared less dense.

Discussion

A number of observations demonstrate that these interphase chromatin fibers represent the DNA — histone complex of chromosomes, and trypsin digestion removes the histone component of this complex (Ris, 1966; Bernhard and Granboulan, 1963; Bastia and Swaminthan, 1967). Therefore, it is justifiable to define the electron dense granules and aggregates left after DNase treatment of chicken erythrocyte chromatin to be primarily histone residue. Furthermore, the dissociation of thick chromatin fibers

witnessed in trypsin treatment indicates that histones act as an adhesive promoting the aggregation of fibers and may play a definitive role in the condensation of heterochromatin.

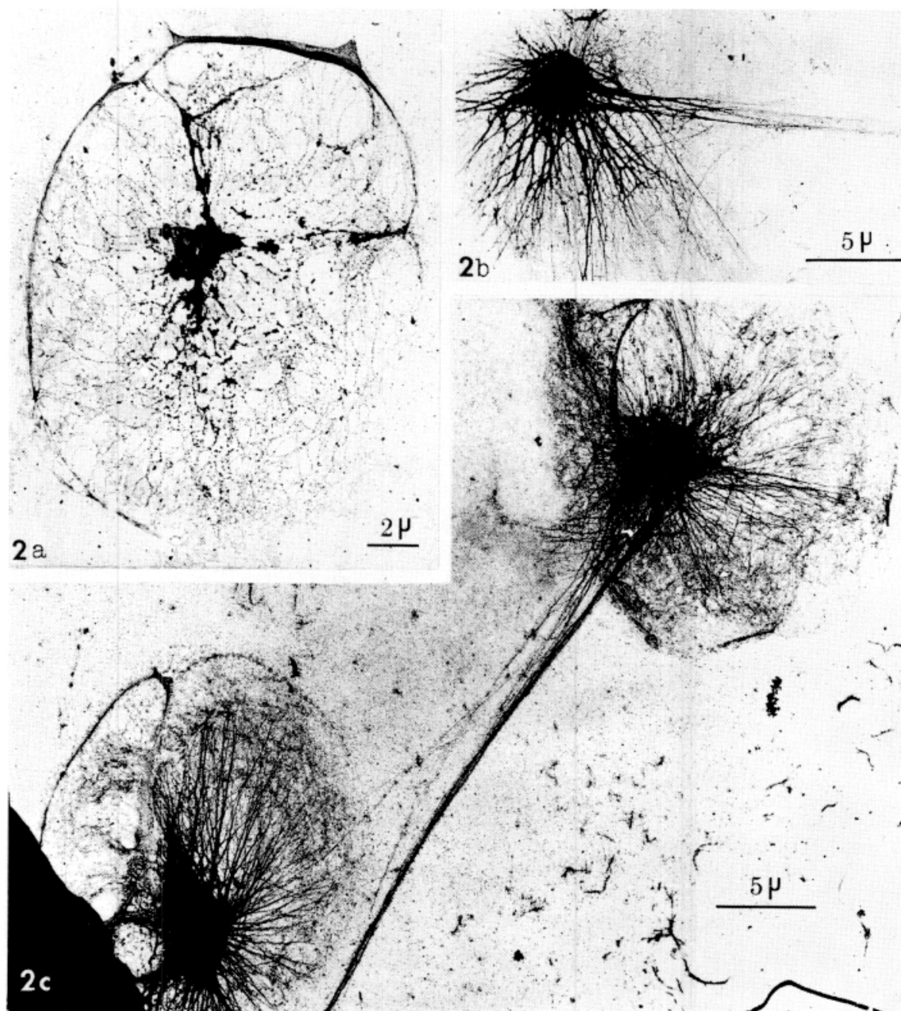

FIGURE 2. (a) A single chicken erythrocyte nucleus after DNase treatment showing ghost fibers and granules and their aggregation into one large central mass representing the remnant of interphase chromocenter. Ghost fibers extent to what is left of the nuclear membrane on the periphry. X 3,460; (b) A single erythrocyte nucleus of chicken treated with RNase. Knobby fibers remain intact with no change in diameter. X 2,590; (c) Two erythrocyte nuclei of chicken digested with trypsin. Fibers lose their knobby appearance and dissociate into a multitude of thinner fibers. X 2,670.

The above described osmotic shock technique used here to release chromatin of intact nuclei into monolayer protein films is much simpler and less time consuming than Gall's Langmuir trough method, especially in the spreading and dehydration steps. It gives reproducible results and should be generally applicable to nuclear fractions of various eukaryotes. Success has already been obtained with macronuclear fraction from the amicronucleate (GL) strain of the ciliate *Tetrahymena pyriformis* and the results are summarized elsewhere (Abdel-Hameed, 1969). Interestingly, both Gall's technique and the one described here are modifications of the water-spreading technique developed originally by Kleinschmidt and his coworkers (Kleinschmidt and Zahn, 1959; Kleinschmidt et al., 1962) to study the fine structure of bacterial and viral genophores.

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