*THE ROLE OF LYSINE-RICH HISTONE IN THE MAINTENANCE OF CHROMATIN STRUCTURE IN METAPHASE CHROMOSOMES**

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Communicated August 13, 1968

Dense masses of chromatin are present in the nuclei of many cells during interphase; dense chromatin is also present in the chromosomes of cells during mitosis. Relatively little RNA synthesis takes place in the dense chromatin of interphase cells,[1, 2] and apparently none at all in the dense chromatin of metaphase chromosomes. It has been shown that chromatin threads of thymus nuclei are bound together into dense masses by lysine-rich histones.[3] In this paper we present evidence that in metaphase chromosomes, too, chromatin threads are held together by lysine-rich histones.

The experimental procedure, as in the previous work, was to remove selectively the lysine-rich histones (which comprise about 20% of the total histone) and then to examine the chromosomes with the electron microscope. Removal of lysine-rich histones loosened the structure of the chromatin in metaphase chromosomes. The special role of lysine-rich histones in binding chromatin threads together was shown by restoring histones to histone-depleted chromosomes. Only the lysine-rich histones caused dense chromatin to reappear, although both arginine-rich and lysine-rich histones combined with histone-depleted chromosomes.

This paper is a sequel to one on chromatin in the interphase nucleus. Some of the procedures followed here are more fully described in the earlier paper.[3]

Materials and Methods.—Preparation of metaphase chromosomes: HeLa cells of the S-3 strain were carried in suspension culture according to standard procedures in Eagle's minimal essential medium supplemented with 5% calf serum, antibiotics, and 4 mM glutamine. The cell line utilized was obtained from Microbiological Associates, and the media from Microbiological Associates and Grand Island Biological Co. Cells were synchronized according to the excess thymidine procedure of Puck.[4] Thymidine was added to exponentially growing cell cultures to 2 mM final concentration. After 24 hr the cells were centrifuged down and resuspended in normal medium. After 5 hr, cell division begins to occur and all the cells in the population divide within the next several hours. At 15 hr from the addition of normal medium, the cells were again exposed to excess thymidine for 24 hr. No cell division at all takes place in the presence of excess thymidine. After removal of thymidine and return to normal medium, a second wave of division, even more highly synchronized than the first, occurs in the cell population. For the experiments described, it was necessary to employ only one cycle of excess thymidine treatment, however.

To obtain metaphase chromosomes, a synchronized cell population was treated with colcemide (0.06 μg/ml) 7 hr after relief of the excess thymidine block. Control cultures were allowed to divide in the absence of colcemide, and in all the experiments cited approximately 100% of the cells underwent division during the next 5–6 hr. Colcemide-treated cells progressed only to metaphase and accumulated at this stage. When harvested, the cell populations thus consisted of up to 90% metaphase cells. The cells were washed in Earle's balanced salt solution and suspended in fresh Earle's containing 0.15% Cemulsol (NTP-12) for 6–10 min at 2°C. This treatment quantitatively released the metaphase chromosomes into the medium. They are shown in Figure 1. The chromosomes were visualized throughout these procedures by staining with aceto-orcein in order to monitor the course of the various preparations. On conclusion of the cemulsol treat-

ment, the chromosomes were suspended in a large volume of cold 0.01 *M* citric acid and harvested by low-speed centrifugation in the cold. Alternatively, the chromosomes could be suspended in Earle's medium, but heavy clumping of the chromosomes usually resulted from this procedure.

Interphase HeLa cell nuclei were prepared by a similar method. The cells were washed in Earle's saline and treated in the cold with 0.15% Cemulsol (NTP-12). They were pipetted vigorously in the cold until the cells were broken and the nuclei released (55–75 min), often with adherent bits of cytoplasm. The nuclei were then resuspended and washed several times in 0.01 *M* citric acid. In other experiments, nuclei were prepared with the use of Tween 80.[5] Cells were suspended in 0.1% Tween 80, and blended in an overhead homogenizer for 25 sec at 4200 rpm in the cold. The nuclei were pelleted and resuspended in 0.25 *M* sucrose–3.3 mM $CaCl_2$, in which they were washed twice by low-speed centrifugation prior to the extraction of histones from them.

When the dense chromatins of interphase chromosomes and interphase nuclei are compared, it is necessary to know the effect, if any, of Cemulsol on the structure of chromatin, as seen in the electron microscope, since Cemulsol was used in the present preparation of metaphase chromosomes but was not used in our previous study of thymus nuclei. Isolated thymus nuclei were, accordingly, treated with Cemulsol. The nuclei were suspended in a solution containing 0.25 *M* sucrose, 0.003 *M* $CaCl_2$, and 0.15% Cemulsol NTP-12. After it had remained in the cold for 45 min with occasional stirring, the suspension was centrifuged, washed in 0.01 *M* citric acid, and prepared for electron microscopy. No effect of exposure to Cemulsol was detected when the electron micrograph of these nuclei was compared with that of nuclei not treated with Cemulsol.

Extraction of histone: Lysine-rich histone was selectively removed from metaphase chromosomes by extraction in a mixture of 0.1 *M* citric acid and 0.125 *M* NaCl. Daly and Mirsky showed that this solvent removes from thymus nuclei all the lysine-rich histone and no more than traces of arginine-rich histone.[6] That this solvent also selectively removes lysine-rich histone from metaphase chromosomes was shown by the following experiments: the total histone was first extracted with 0.2 *N* HCl, precipitated in acetone, and then subjected to electrophoresis on cellulose polyacetate at pH 9; an electrophoretic pattern like that of thymus histone was obtained—two bands, of which the one due to the arginine-rich histones contained four times as much protein as the lysine-rich histone band. Another, and equal, mass of metaphase chromosomes was extracted with 0.2 *M* citric acid and 0.125 *M* NaCl and subsequently with 0.2 *N* HCl. Only the protein extracted in 0.2 *N* HCl was collected (that removed by citric acid was so slight that it was difficult to collect) and examined electrophoretically. Only the band of arginine-rich histone was present, an indication that the lysine-rich histone had been extracted by 0.1 *M* citric acid with 0.125 *M* NaCl.

Effects of Selective Histone Removal with Citric Acid.—Chromosomes isolated in 0.01 *M* citric acid have highly condensed chromatin (Figs. 3 and 5) as do metaphase chromosomes in the intact cell (Fig. 2). When the citric acid concentration is raised to 0.10 *M* (in the presence of 0.125 *M* NaCl), all the lysine-rich histone is extracted and there appears a network of fibrils instead of clumps of chromatin (Figs. 4 and 6). When the citric acid concentration is raised to 0.10 *M in the absence of NaCl*, all the lysine-rich histone is extracted but the change in state of the chromatin does *not* occur.

In our previous experiments on removal of lysine-rich histone from thymus nuclei, extraction in 0.10 *M* citric acid was done in presence of 0.125 *M* NaCl, and a loosening of clumped chromatin was observed. These experiments on thymus nuclei were repeated and it was found that when extraction with 0.10 *M* citric acid was done in the absence of 0.125 *M* NaCl, the change in state of chromatin did not occur. Extraction of lysine-rich histone by 0.10 *M* citric acid was com-

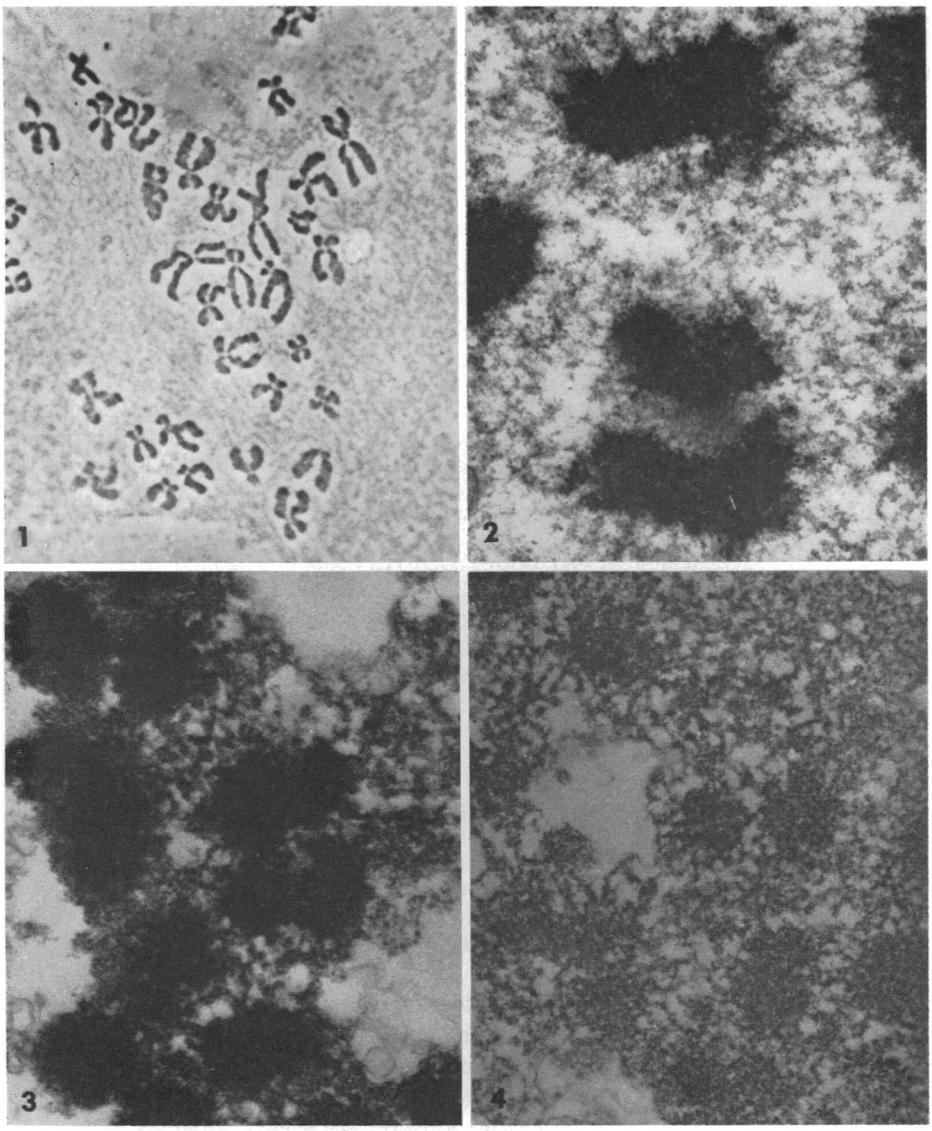

FIG. 1.—Isolated HeLa metaphase chromosomes. They were fixed in 3:1 ethanol:acetic acid, stained with aceto-orcein, and photographed under phase contrast. ×1229.

FIG. 2.—Metaphase chromosomes of HeLa cells in the intact cell. This preparation, as all the material for electron microscopy, was fixed in 0.5% osmium tetroxide in "incubation medium" at 4°C for 2 hr, dehydrated in ethanol, and embedded in Finck's[9] Epon mixture A. Sections were stained with aqueous uranyl acetate. ×28,800.

FIG. 3.—Isolated chromosomes in 0.01 *M* citric acid. The lysine-rich histone still present in the chromosomes. ×28,800.

FIG. 4.—Isolated chromosomes extracted by a solvent of 0.10 *M* citric acid and 0.125 *M* NaCl. Lysine-rich histone has been extracted. ×28,800.

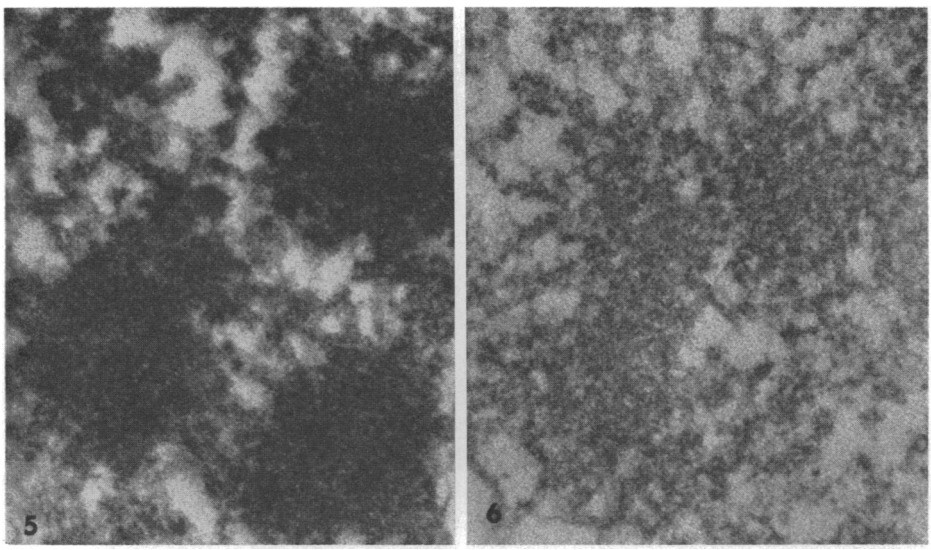

FIG. 5.—Same as Fig. 3, but at a magnification of 67,200.
FIG. 6.—Same as Fig. 4, but at a magnification of 67,200.

plete whether or not 0.125 *M* NaCl was present. Furthermore, the electrophoretic patterns at pH 9 on cellulose polyacetate of the extracted histones were the same whether or not 0.125 *M* NaCl was present.

Effects of Adding Lysine-Rich and Arginine-Rich Histones to Histone-Depleted Chromosomes.—Total histone was removed from chromosomes by extraction in the cold with a solution of 80 per cent 0.18 *N* HCl. An electron micrograph (Fig. 6) shows that these chromosomes consist of a loose network of fibrils. To these chromosomes, suspended in a cold sucrose and phosphate buffer medium (1.0 ml of 0.25 *M* sucrose–3 mM $CaCl_2$, 0.5 ml of 0.1 *M* sodium phosphate–0.25 *M* sucrose buffer (pH 6.75), 0.4 ml of 0.1 *M* glucose solution containing 3.75 mg NaCl and 4.19 mg $MgCl_2 \cdot 4H_2O$ per ml, and 0.1 ml H_2O), an excess of histone dissolved in the same medium was added. The mixture was stirred gently in the cold for 15 minutes and then centrifuged. The chromosomes were finally resuspended in the sucrose medium before being fixed for electron microscopy.

The lysine-rich histone added to the histone-depleted chromosomes was prepared from calf thymus nuclei by extraction with 0.1 *M* citric acid (*not* containing 0.125 *M* NaCl). Traces of arginine-rich histone in the extract were removed by precipitation at pH 10.6. To prepare arginine-rich histone, the citric acid nuclear residue was extracted with 0.2 *N* HCl. Electrophoresis on strips of cellulose polyacetate at pH 9 showed that the lysine-rich and arginine-rich histones had been completely separated from each other.

Addition of lysine-rich histone to chromosomes completely depleted of histones caused the loose fibrils (Fig. 7) to form dense masses of chromatin (Fig. 8). Arginine-rich histone, though it combines with the histone-depleted chromosomes, left them as loose assemblies of fibrils (Fig. 9).

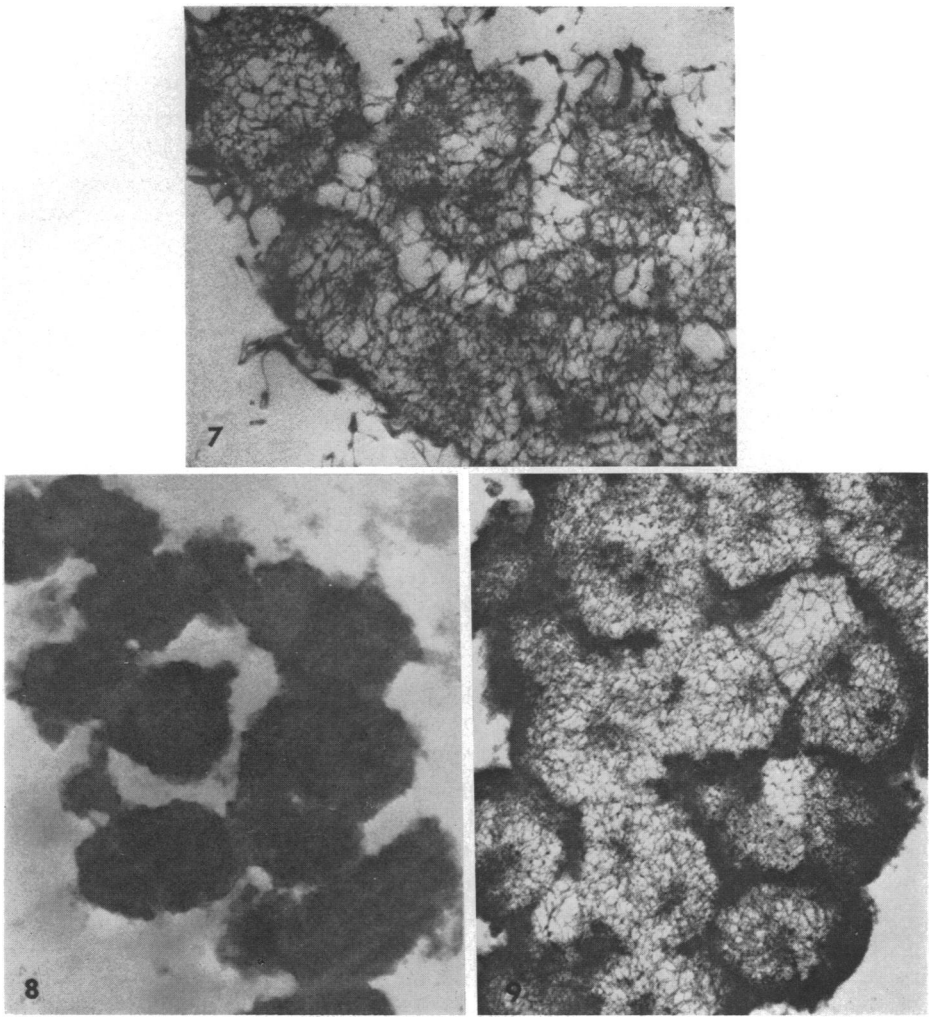

FIG. 7.—Isolated chromosomes, from which all histones have been extracted. ×28,800.
FIG. 8.—Histone-depleted chromosomes to which lysine-rich histone has been added. ×28,800.
FIG. 9.—Histone-depleted chromosomes to which arginine-rich histone has been added. ×28,800.

Discussion.—Both the experiments on removal of lysine-rich histone from chromosomes and those in which histones are restored to chromosomes provide evidence that lysine-rich histones cross-link fibrils containing DNA. The role of sodium chloride in the medium used for the selective extraction of lysine-rich histone is obscure. The possibility remains that some significant material in addition to lysine-rich histone is extracted. Addition of lysine-rich histone to histone-depleted chromosomes shows more unequivocally that lysine-rich histone can cross-link the DNA-containing fibrils of chromatin, irrespective of whether or not some material other than lysine-rich histone was extracted from chromatin by 0.1 *M* citric acid in presence of 0.125 *M* NaCl. It would be of interest to frac-

tionate the lysine-rich histone preparation and test the subfractions for their capacity to cross-link fibrils of chromatin.

The amount of chromatin that is in a condensed form varies in a given cell with the stage of development and also with the phase of the mitotic cycle. An example of a developmental change is the marked increase in quantity of condensed chromatin that is observed during the development of polymorphonuclear leucocytes.[7] Examples of changes associated with cell division are the loosening of chromatin in spots where DNA synthesis is occurring in the interphase of proliferating cells[8] and the condensation of chromatin in prophase. Recognition of the role of lysine-rich histone in the condensation of chromatin raises some questions: since lysine-rich histone can probably combine with DNA either alongside one fiber or cross-linking several fibers, how is such selective combination determined and how is the selective combination directed to a particular portion of the chromatin?

Summary.—In the metaphase chromosomes, as well as in interphase nuclei, lysine-rich histone cross-links DNA-containing fibrils to form condensed chromatin.

* This research was supported in part by U.S. Public Health Service grant GM-04919.

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[1] Littau, V. C., V. G. Allfrey, J. H. Frenster, and A. E. Mirsky, these PROCEEDINGS, **52**, 93 (1964).

[2] Hsu, T. C., *Exptl. Cell Res.*, **27**, 332 (1962).

[3] Littau, V. C., C. J. Burdick, V. G. Allfrey, and A. E. Mirsky, these PROCEEDINGS, **54**, 1204 (1965).

[4] Puck, T. T., *Science*, **144**, 565 (1964).

[5] Fisher, H. W., and H. Harris, *Proc. Roy. Soc. (London)*, **B262**, 521 (1962).

[6] Daly, M. M., and A. E. Mirsky, *J. Gen. Physiol.*, **38**, 405 (1955).

[7] Bainton, D. F., and M. G. Farquhar, *J. Cell Biol.*, **28**, 277 (1966).

[8] Hay, E. D., and J. P. Revel, *J. Cell Biol.*, **16**, 29 (1963).

[9] Finck, H., *J. Biophys. Biochem. Cytol.*, **7**, 27 (1960).