STABILITY OF NEWLY SYNTHESIZED NUCLEOPROTEIN COMPLEXES

M. Michael Thaler and Claude A. Villee

Department of Biological Chemistry, Harvard Medical School and the Laboratory of Reproductive Biology, Boston Hospital for Women, Boston, Massachusetts.

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In the course of experiments with purified, solubilized chromatin the possibility arose that nucleic acid-protein complexes which are formed in cells preparing for mitosis are differently affected by the procedures employed for purification and solubilization than are stabilized complexes from the inactive phase of the cell cycle. Since chromatin is usually solubilized by means of shearing or blending forces (Marushige and Bonner, 1965; Zubay and Doty, 1959), we examined the effects of shear on purified chromatin preparations from normal rat liver and from livers following partial hepatectomy. Animals were killed 20 hours after the hepatectomy, at the period of maximal DNA and histone synthesis (Grisham, 1962; Umana, 1963). We found that DNA and nucleoprotein appear to be synthesized and complexed in small units before incorporation into chromosomes and that these newly formed complexes are readily disrupted by shearing forces.

METHODS

Male Sprague-Dawley rats, weighing 150-200 gm, were injected with 50 uc of ^{3}H-thymidine (S.A. 6 mc/mmole) two hours before partial hepatectomy (Higgins and Anderson, 1931). The portions of liver removed

were frozen on dry ice and served as normal controls. Animals were killed 20 hours after operation, and the regenerating livers were removed and treated as above. Chromatin was prepared from 2 gm of frozen tissue. The liver was homogenized in 20 ml of 0.05 M NaCl - 0.016 M Na EDTA solution, pH 8.0, for 1 min at 90 V and 4 min at 50 V in a Waring blender. 0.1 ml of 2-octanol was added to prevent foaming. The homogenate was filtered through four layers of surgical gauze and centrifuged at 1500 x g for 15 min. The pellet was washed successively in 10 ml of saline-EDTA solution and 10 ml of 0.05 M tris buffer, pH 8.0. Each wash was followed by centrifugation at 1500 x g for 15 min. The gelatinous pellet was suspended in 5 ml of 0.05 M tris buffer, pH 8.0, layered over 25 ml of 2.0 M sucrose in tris buffer solution, and a rough gradient was established along the upper two-thirds of the tube. The suspension was centrifuged in the SW 25 rotor at 50,000 x g for 5 hours. The clear pellet of chromatin was dialyzed overnight against 100 volumes of tris buffer, 0.01 M, pH 8.0, suspended in 10 ml of the same buffer and blended in a Lourdes homogenizer at 90 V for 90 sec. The sheared chromatin solution was cleared by centrifugation at 10,000 x g for 30 min. To prepare unsheared chromatin, the pellet obtained after sucrose gradient centrifugation was suspended in 0.01 M tris buffer by means of 3-4 strokes of a loosely fitting Teflon pestle in homogenizer.

Aliquots of chromatin suspension or sheared chromatin solution were placed on a 25.5 x 3 cm column of Sephadex G-75, and eluted from the column with phosphate buffer, 0.1 M, pH 7.2. Blue dextran (M.W. 2,500.000) and DNP-glycine (M.W. 250) were used as markers. 3 ml fractions were collected and optical densities determined at 260 mu and 280 mu. Protein content of each fraction was assayed by the method of

Lowry et al (1951), and DNA determined with the diphenylamine reaction (Burton, 1956). 1 ml aliquots of each eluate fraction, reduced to 0.1 ml by slow evaporation in counting vials, were dissolved in .5 ml of hyamine, 10 ml of toluene fluor was added, and the radioactivity determined by liquid scintillation counting.

RESULTS

Elution profiles of unsheared chromatin from normal and regenerating liver are shown in Fig. 1. The washout peak contained most of the nucleic acid. The specific activity of DNA which was excluded from the column was 3,300 cpm/mg in normal, and 12,700 cpm/mg in regenerating liver, respectively.

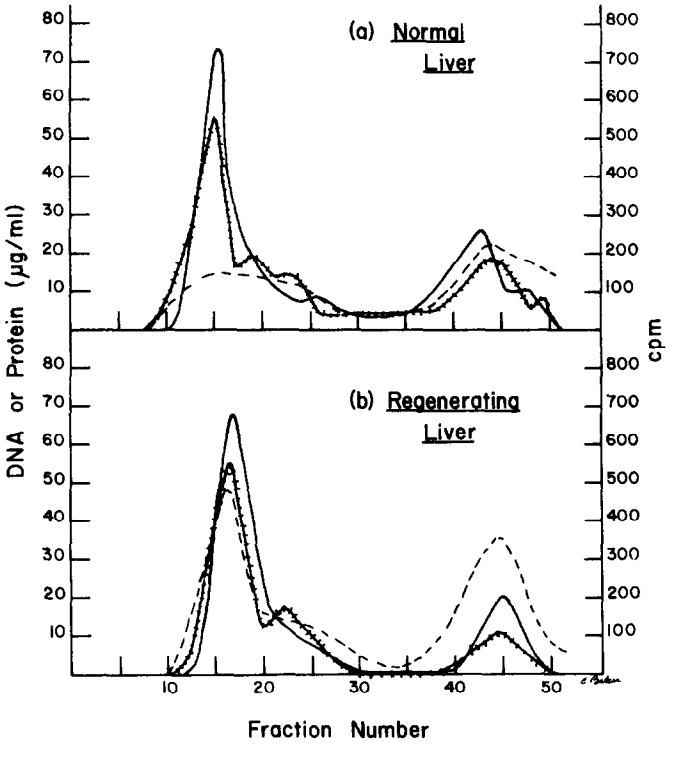

Fig. 1. Elution profiles of unsheared chromatin from normal and regenerating liver. The solid line indicates amount of protein. The hatched line indicates amount of DNA, and the dashed line indicates amount of radioactivity.

A single peak emerged from the column in the molecular size range between 3000 and 5000. Since the preparation was carefully dialyzed, it is unlikely that the strong radioactivity in this region was due to unincorporated thymidine or thymidine phosphates. The specific activities of the DNA in this fraction of chromatin were 13,000 cpm/mg and 27,300 cpm/mg in normal and regenerating liver, respectively. Shearing did not appreciably affect the number, size, or specific activity of these oligonucleotides (Fig. 2A). In contrast, the protein which was eluted with the oligonucleotides was markedly reduced by the shearing process (Fig. 2B). In chromatin suspended without shearing, the ratio of protein to DNA in the

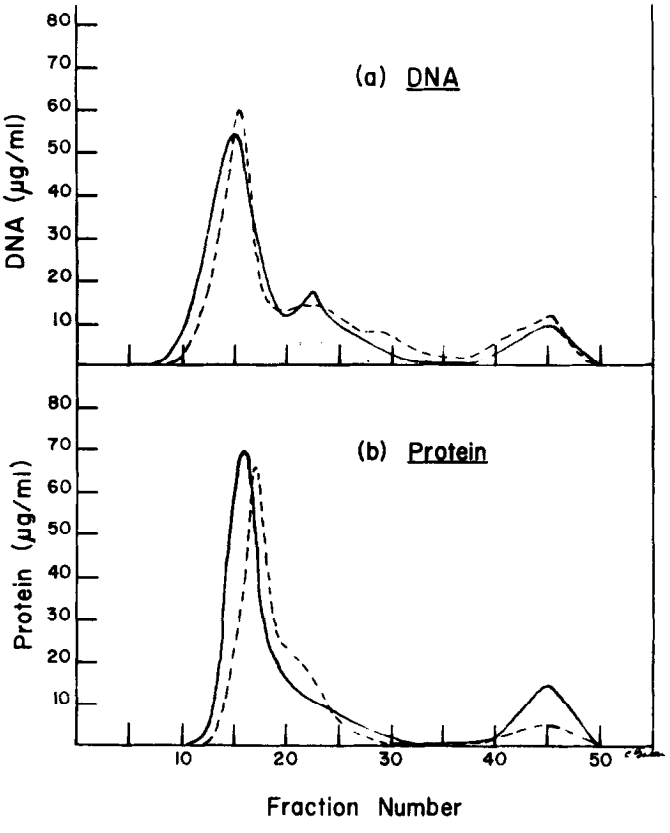

Fig. 2. Elution profiles of sheared (dashed line) and unsheared (solid line) chromatin from regenerating liver.

region of the peak was 1.5; when shearing forces were applied for 90 sec., the ratio dropped to 0.44. The ratio of protein to DNA in the bulk chromatin which was excluded by the column was 1.3 before and 1.1 after shearing.

DISCUSSION

A column of Sephadex G-75 has an exclusion limit of 50,000. Most of the chromatin suspended by gentle homogenization or solubilized by shearing forces is excluded from this column. However, a small portion of the chromatin is composed of fragments of 3000 to 5000 molecular weight. These fragments appear to be oligonucleotides complexed to protein which elute from the column in a single peak.

The specific activity of the DNA in this small fraction of chromatin was 3 to 4 times higher than in the bulk chromatin containing complexes of high molecular weight. This indicates that the oligonucleotide fraction contains a large proportion of the nascent DNA, a finding which is particularly apparent in chromatin from regenerating liver. Since the high specific activity of these fragments of DNA was not affected by shearing forces, they cannot be products of random breakage.

These newly synthesized oligonucleotides may be subunits loosely associated with the structural proteins of chromosomes and easily separated from them, or they may be new DNA-protein complexes not yet incorporated into chromosomes. In either case, the loss of protein from this fraction caused by shearing forces which did not affect large molecular complexes shows that these newly formed protein-DNA complexes are particularly fragile.

These findings indicate that DNA may be synthesized and complexed with protein before forming an integral part of the genome. The greater sensitivity to shear observed with newly synthesized chromatin suggests

that protein-DNA complexes in cells preparing for mitosis are less stable than in cells which have ceased synthesizing DNA.

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