EXAMINATION OF THE NUCLEOHISTONE FROM MOUSE LIVER AND INTESTINE FOR RNA COVALENTLY LINKED TO HISTONE*

BY S. L. COMMERFORD AND N. DELIHAS

DIVISION OF MICROBIOLOGY, MEDICAL RESEARCH CENTER,
BROOKHAVEN NATIONAL LABORATORY, UPTON, NEW YORK

Communicated by Donald D. Van Slyke, October 6, 1966

Huang and Bonner have recently reported that histones associated with DNA in chromosomes of pea bud cells are covalently linked to RNA. They have suggested that these molecules, which combine the specificity of RNA with the gene inhibitory activity of histones, are the agents through which cells, in general, control gene expression.¹ To test this hypothesis we have examined mouse liver and intestine for the presence of these RNA-histones. We adopted the following criteria: (1) the RNA-histones must be present in a deoxyribonucleohistone preparation, absorb in the ultraviolet at 260 $m\mu$, and have a buoyant density in CsCl of less than 1.56; (2) the RNA must become acid-soluble and nonsedimentable after digestion in alkali.

Nucleohistone was prepared from livers or intestines removed from mice within 2 min after death and frozen in liquid nitrogen. The tissue was powdered in a Teflon mortar and homogenized with 20–25 vol phosphate-buffered saline (0.12 M NaCl, 0.008 M K_2HPO_4 , 0.002 M KH_2PO_4 , pH 8). After removal of coarse connective tissue fragments with a stainless steel screen, the homogenate was centrifuged, the sediment washed twice in the same volume of phosphate-buffered saline and once in the same volume of EDTA-buffered saline (0.12 M NaCl, 0.01 M Na_2EDTA brought to pH 8 with NaOH). The nucleohistone was extracted from the washed sediment by homogenizing in 0.001 M EDTA, pH 8 in one fifth the vol used for the washes. This was centrifuged for 30 min at 10,000 g and the sediment discarded.

The median sedimentation coefficient for this nucleohistone at a concentration of 0.8 mg/ml in 0.001 M EDTA at 25°C is 20S, and over 98 per cent has a sedimentation velocity greater than 4S (Spinco model E ultracentrifuge, ultraviolet optics). The liver nucleohistone preparation contains an impurity which floats at densities greater than 1.08. It is probably microsomal membranes and represents about 30 per cent of the dry weight of liver nucleohistone. None is seen in intestinal nucleohistone. After removal of microsomal membranes the nucleohistone solution has a typical nucleic acid UV-absorption spectrum; from the absorption at 260 $m\mu$ it is estimated that 40 per cent of the dry weight represents nucleic acid.

We were consistently unable to find RNA-histones in these preparations. The results of a typical experiment are as follows: 5 ml of a solution containing 5 mg liver nucleohistone in 2.6 M CsCl ($\rho = 1.34$) was centrifuged 40 hr at 35,000 rpm, 5°C in an SW39 rotor. The thin film of microsomal membranes floating on the surface was discarded and all the gradient with a density of less than 1.4 was collected. This had an absorption maximum at 275 $m\mu$ and a minimum at 255 $m\mu$, and its absorption at 260 $m\mu$ represented 2.6 per cent of the total present. After digestion at 37°C in 0.3 N KOH and precipitation in ice-cold 0.2 M $HClO_4$, the

absorption at 260 $m\mu$ represented 0.36 per cent of the total originally present. The UV-absorption spectrum was typical of degraded nucleic acid. Considering the hyperchromic effect of degradation, we consider that less than 0.2 per cent of the total absorption at 260 $m\mu$ in our nucleohistone preparations could represent RNA-histone having a density less than 1.4. We were also unable to find RNA-histone having densities greater than 1.4. When a solution containing 0.5 mg/ml nucleohistone and 45 per cent CsCl by weight was centrifuged at 44,770 rpm, 25°C in a Spinco model E ultracentrifuge, no UV-absorbing band appeared in photographs taken after 25 hr. Bands would have been visible under these conditions if as little as 0.3 per cent of the nucleic acid had a density between 1.40 and 1.56, the density expected for RNA-histone containing from 25 to 50 per cent RNA by weight.

It is possible that we have failed to observe RNA-histone because we did not extract it. However, according to the diphenylamine reaction of Dische as modified by Burton,² our nucleohistone preparation containing over 50 per cent of the DNA in the original tissue and over 80 per cent of the DNA in the sediment after the final wash; also some of the losses were random.

It is also possible that the RNA-histone became degraded before we could detect it. This does not seem likely since all operations were carried out between 0 and 5°C, the total preparation time was only 4–6 hr, only fresh preparations were analyzed, and the deoxyribonucleohistone sedimentation pattern was unchanged after storing solutions of nucleohistone for several weeks which suggests that no significant proteolytic DNase or RNase activity was present. If it had been destroyed during centrifugation by ribonuclease activity, this would have been detected as an increase in nonsedimented UV-absorbing material, but this was never seen.

We have also attempted to detect RNA-histone by means of P^{32} incorporation. Nucleohistone was prepared from mice given 10 μc P^{32} 16 hr previously, and CsCl was added to give a density of 1.28 at 25°C. After centrifuging for 21 hr in an SW39 rotor at 35,000 rpm, the floating film, the supernatant, and the pellet were analyzed for P^{32} with a gas-flow counter. About 80 per cent of the activity was in the floating film; less than 0.6 per cent of the activity was found in the supernate.

Our nucleohistone preparations do contain RNA. Judging by the amount of nucleotides recovered after KOH digestion, 3–4 per cent of the total nucleic acid present is RNA. The nucleotide composition of RNA associated with nucleohistone resembles that of ribosomal RNA; it has the following composition: CMP, 27.4 ± 4.5 ; AMP, 17.5 ± 3 ; GMP, 36.4 ± 2.9 ; UMP, 18.7 ± 2.8 . These values are expressed as moles/100 moles nucleotide $\pm$ standard deviation and represent the average of seven determinations. Nucleotides were separated by thin-layer electrophoresis at pH 3.1³ after neutralization of the KOH digest with PCA. Since Huang and Bonner reported the presence of 28 per cent dihydro-uridylic acid in their RNA-histone, we examined our nucleohistone for this nucleotide. Tests for ribosyl phosphate of β -ureidopropionic acid (open ring form of dihydrouridylic acid) in the KOH digests of nucleohistones were made with acidified *p*-dimethylaminobenzaldehyde.⁴ After electrophoresis, thin-layer plates were sprayed with the reagent and examined for the development of yellow color. No color was found in regions where β -ureidopropionic acid ribosyl phosphate migrates.

Under these experimental conditions, the presence of β -ureidopropionic acid ribosyl phosphate would have been detected if dihydrouridylic acid represented 10 per cent or more of the total RNA nucleotides. On the other hand, intense color developed with two unidentified bands having an electrophoretic R_f (relative to U_p) of 0.16 and 1.4. These components do not appear to be acid breakdown products of the open-ring form of dihydrouridylic acid since no degradation has been observed at pH 3.1, 0°C; also the acid degradation product of β -ureidopropionic acid ribosyl phosphate giving a positive ureido test with the reagent migrates with an $R_f = 0.26$. The two ureido groups were not further identified.

The presence of RNA-histone in rat liver has recently been reported.⁵ This does not necessarily conflict with our results since the amount found was not stated, and their RNA-histone (obtained by extracting whole nuclei with 2 *M* NaCl), may be nonchromosomal in origin.

Huang and Bonner found that pea nucleohistone contained over 100 RNA-histone nucleotides/1000 DNA nucleotides. We have found that there can be no more than 2 RNA-histone nucleotides/1000 DNA nucleotides in mouse liver or intestine. This amount is certainly insufficient to allow each histone to be covalently linked to RNA as in pea nucleohistone and probably also insufficient to control genetic activity. It seems likely that the RNA-histones of peas are performing a more special function.

We thank Dr. L. D. Hamilton for stimulating this work, and Mr. W. Ogden and Mrs. J. Bertman for valuable technical assistance.

* This work was supported by the U.S. Atomic Energy Commission.

¹ Huang, R. C., and J. Bonner, these PROCEEDINGS, **54**, 960 (1965).

² Burton, K., *Biochem. J.*, **62**, 315 (1956).

³ Tometsko, A., and N. Delahas, *Anal. Biochem.*, in press.

⁴ Fink, R. M., R. E. Cline, C. McGaughey, and K. Fink, *Anal. Chem.*, **28**, 4 (1956).

⁵ Benjamin, W., O. A. Levander, A. Gellhorn, and R. H. DeBellis, these PROCEEDINGS, **55**, 858 (1966).