Antagonistic Action of Exogenous Histone and RNA in Mouse Ascites Cells[1] (36437)

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Mouse ascites cells are incapable of synthesizing liver specific protein, serum albumin. When incubated with liver-RNA, they acquire the capability to produce albumin (1, 2). Apparently exogenous-RNA acts in the ascites cells as "template" in new protein synthesis (2). However, studies on uptake of RNA shows that silver grains of autoradiograms are evenly distributed in the RNA-treated cells (3) or more in nucleus than in cytoplasm (4, 5). In view of albumin synthesis in cytoplasm, those RNA molecules that enter the nucleus would have a different function, *e.g.*, altering the nuclear activity (6). Histone has long been known to influence the template activity of DNA. Treatment of living tissue with it results in the inhibition of growth and differentiation (7–10). Active workers in this field are inclined to believe that exogenous histone enters the nucleus and directly participates in the formation of chromatin complex (7, 9). According to the latter view, histone would have to be found predominantly in nucleus of histone-treated cells. If, on the other hand, the function of exogenous RNA in the recipient cells were antagonistic to that of histone and thus capable of lifting the histone inhibited RNA synthesis, it would also act on DNA-dependent RNA synthesis. The aim of this study was to provide data showing that: (a) histone enters the nucleus of ascites cells in medium with pH 7.3–7.6, (b) histone represses RNA synthesis to approximately one third of the control, and (c) RNA stimulates RNA synthesis and, besides, is capable of raising the histone-depressed RNA synthesis to a level close to the RNA-treated series. Conversely, histone represses the RNA-stimulated RNA synthesis to a level close to the histone-treated series.

Materials and Methods. Nelson mouse ascites cells were obtained from Professor John B. Nelson of Rockefeller University. This strain was maintained through weekly transfer in Princeton mice. Six to 8 mice were kept in one cage. When ascites fluid was bloody, red blood cells were removed by several washings with physiological saline and differential centrifugation. The packed cells were suspended in 10 vol of saline. Each mouse received intraperitoneal injection of 0.1 ml. The remaining cell suspension was used for experiments described below.

The ^{125}I, ^{3}H-uridine and ^{3}H-uridine triphosphate used were products of New England Nuclear (Boston), actinomycin D and puromycin were purchased from Nutritional Biochemical (Cleveland), ribonuclease was from Worthington and poly I-C from Miles Laboratory.

Isolation of RNA. Calf liver was purchased from Cross Brothers Meat Packing House in Philadelphia. Liver was excised immediately after the slaughtering of the calf. It was sliced and brought back to the laboratory in ice-cold sucrose (0.25 *M* sucrose and 0.003 *M* $MgCl_2$) within 30 min. All operations were performed in a cold room (2).

One hundred grams of liver were chopped into fine pieces by scissors and blended in a

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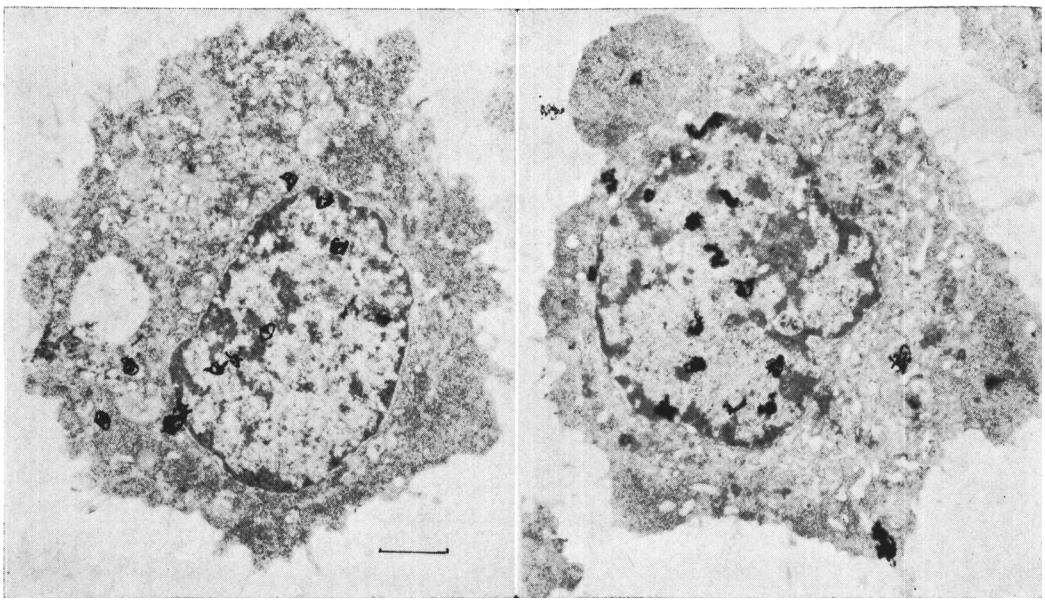

FIG. 1. High resolution autoradiograms of two ascites cells: (left) an even distribution of silver grains, and (right) silver grains predominantly in the nucleus.

Waring Blender with 6 vol of buffered saline (0.15 *M* NaCl, 0.1 *M* Na-acetate, and 0.6% diethyl pyrocarbonate, pH 5–5.2). The homogenate was filtered through 4 layers of cheesecloth. To the filtrate an equal volume of water-saturated phenol (redistilled) was added and mixed with Lourdes mixer at 60 V for 5 min. After centrifugation at 1700 rpm for 30 min, the top aqueous layer was removed by aspiration. The interphase and phenol layer was washed with 0.5 vol of the buffered saline and centrifuged. The top layer was combined with the above. The combined supernatant fluid was mixed with 0.5 vol of phenol at 40 V for 5 min. To the top aqueous layer K-acetate was added up to 2% and then 2.5 vol of alcohol. The precipitate was collected by centrifugation and redissolved in saline. Glycogen was removed by high speed centrifugation (20,000 rpm for 20 min). Phenol was removed by 3–5 washings with ether. The latter was evaporated under negative pressure with the aid of magnetic stirring. The RNA solution was washed twice with alcohol before being stored in deep freeze (—20°). The use of fresh RNA was preferred in our studies. However, the biological activity of the RNA stored in 2 vol of alcohol at —20° (2 weeks) was practically the same as the newly isolated.

The UV spectrum of the above RNA was typical of nucleic acids. Diphenylamine test for DNA was negative. Lowry test for protein yielded an amount less than 1%. Orcinol color test was positive. The amount of RNA calculated from determinations of ribose-P was in good agreement with that calculated from absorbance at 260 nm. This equivalence indicated that RNA alone was responsible for the UV absorbed material. Sucrose density gradient centrifugation of this RNA gave 3 peaks corresponding to the RNAs with 28S, 18S and 4S.

RNA was separated by 1 *M* NaCl into high and low molecular weight fractions. The ratio between the high and low was usually 9:1.

An aliquot of liver was homogenized in sucrose solution (0.32 *M* sucrose and 0.003 *M* $MgCl_2$). The homogenate was filtered through 4 layers of cheesecloth and then 2 layers of flannel. The filtrate was centrifuged at 1600 rpm for 30 min. The sediment was washed twice with sucrose and then used for isolation of nucleolar RNA (nRNA) according to the method of Penman (11, 12).

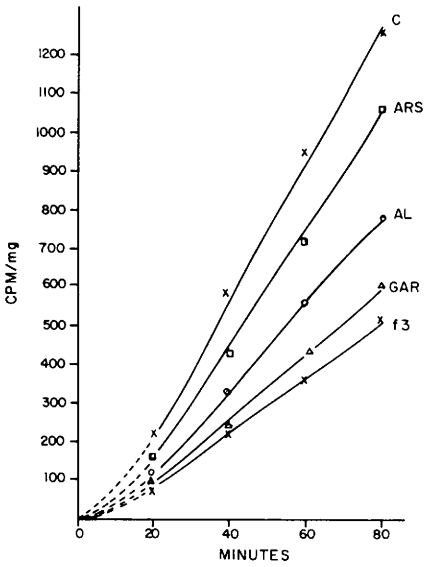

FIG. 2. The effect of histone subfractions (200 μg/ml) on the incorporation of ^{3}H-uridine triphosphate (1 μCi/ml) into acid-insoluble material (CPM/mg dry weight). (C) control; (f3 and GAR) arginine-rich histones.

Iodination of histone. The histone prepared by Professor Wang was employed for iodination with ^{125}I. For detailed procedure, see Ref. (13).

High resolution autoradiography. Ascites cells were suspended in Krebs–Ringer solution with 18 amino acids (14). To the cell suspension 100 μg/ml of ^{125}I-histone was added. The pH of this mixture was 7.4. It was incubated at 37° with frequent stirring for 1 hr. Unabsorbed histone was removed by 2 washings with cold saline. Color exclusion test for viability showed that over 95% of cells were not stained. Viable cells were fixed in glutaraldehyde. The procedure for high resolution autoradiography was published elsewhere (3).

Incubation with ^{125}I-histone was also done at pH 6.9. These cells were separated by differential centrifugation into nuclear (N) and cytoplasmic (C) components. The radioactivity of N/C was recorded by Packard Tri-Carb scintillation spectrometer.

RNA synthesis. ^{3}H-Uridine and ^{3}H-uridine triphosphate were used as precursors for RNA synthesis. The ascites cells were pretreated with RNA (10–100 A_{260}/ml) histone (100–200 μg/ml), poly I-C (10–20 μg/ml) or RNAse-digested RNA in Krebs–Ringer solution for 2 hr at room temperature. They were packed and resuspended in incubation mixture (14) (1:50 by volume) containing ^{3}H-uridine (1 μCi/ml) or ^{3}H-uridine triphosphate (1 μCi/ml) and RNA or histone. Actinomycin (1 μg/ml) or puromycin (1 μg/ml) was added as specified. Incubation was carried out at 37° in a Dubnoff metabolic shaker (50 cycles/min). Cell viability was recorded before and after incubation. At a predetermined time interval, an aliquot was removed and mixed thoroughly with equal volume of ice-cold 10% trichloroacetic acid (TCA). The acid-insoluble material was collected by centrifugation and washed 4 more times with 5% TCA. The sediment was dehydrated by going through 95% alcohol, 3 times of alcohol–ether (3:1) at 62°, once alcohol–ether–chloroform (2:1:1) and ether. RNA component of the dried nucleoprotein was hydrolyzed in 0.3 *N* NaOH at 37° for 18 hr. The solution was neutralized and the supernatant fluid was counted by Packard Tri-Carb scintillation spectrometer.

Results. Uptake of ^{125}I/histone. For the uptake of macromolecules, it is of prime importance to get rid of: (a) the extracellular material by washing the cells with saline at the end of incubation, and (b) enzymatic breakdown products of fixed tissue by several

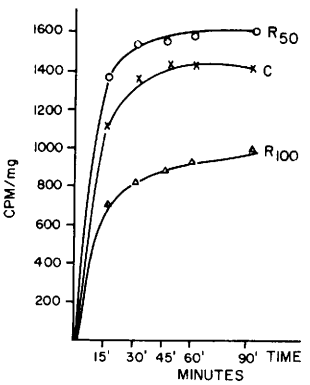

FIG. 3. The effect of RNA concentration on the incorporation of ^{3}H-uridine (1 μCi/ml) into ^{3}H-RNA. (R_{50} and R_{100}) series treated with RNA, 50 and 100 A_{260}/ml, respectively, and (C) is control.

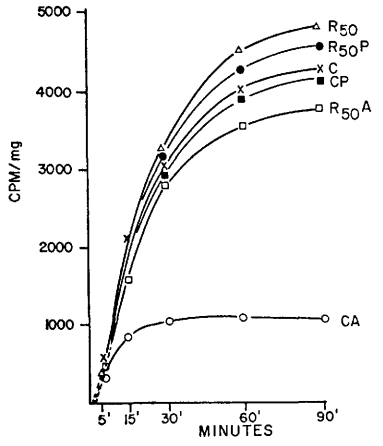

FIG. 4. The effect of actinomycin D (A, 1 μg/ml) and puromycin (P, 10 μg/ml) on the RNA-treated (50 A/ml) and control series (^{3}H-uridine as precursor).

changes of 70% alcohol. Intracellular distribution of ^{125}I-histone can then be localized by autoradiography. Figure 1 shows high resolution autoradiograms of two ascites cells: the left has an even distribution of silver grains and the right has them predominantly in the nucleus. Ascites cells do not utilize iodine metabolically. Therefore, the presence of silver grains can only be due to intracellular distribution of ^{125}I-histone. It may be added that intracellular distribution of histone was shifted by the change of pH. The ^{125}I-histone-treated cells were fractionated into nuclear (N) and cytoplasmic (C) components. The ratio of radioactivity of N/C was less than 1 at pH 6.9, but greater than 1 at pH 7.3–7.6.

Under the following headings, each experiment was done in duplicate and repeated 3–5 times using different lots of RNA preparations. Only those data reproducible are given in the following figures.

RNA synthesis of histone-treated cells. Arginine-rich (GAR), lysine-rich (AL) and slightly lysine-rich (ARS) subfractions of histone were received from Dr. Bush and arginine-rich histone (f3) from Dr. Johns. The nuclear accumulation of histone made the nucleus the likely site for its action. Since removal of histone from nuclei resulted in an increase in RNA synthesis (15) and since addition of histone to DNA-containing reaction mixture decreased RNA synthesis (16), one would expect that histone in living cells also inhibits RNA synthesis. Figure 2 illustrates the inhibitions of RNA synthesis by 4 subfractions of histone. Cell injury was noted in experiment using histone more than 50 μg/ml (17). In our hands, however, viability of cells from all series was good, *i.e.*, within the range of 85–95%.

RNA synthesis of RNA-treated cells. Figure 3 depicts the effect of exogenous RNA on ascites cells. In the range of 10–50 *A*/ml, RNA stimulated RNA synthesis up to 114–150% of the control (Figs. 3 and 6). With 100 *A*, it was inhibitory. The increase was abolished by actinomycin D, but not by puromycin (Fig. 4).

Biological potentialities of liver RNA and its subfractions were compared. The low molecular weight fraction was found to be most potent, thus making it likely that liver RNA owed its function to the low molecular component (Fig. 5). The maximal activity of the low molecular RNA ever recorded was 150% of the control (Fig. 6). This was still below the level of nRNA-induced RNA synthesis (Fig. 7). However, synthetic poly I-C was unable to stimulate RNA synthesis (Fig. 8).

Antagonistic action of RNA and histone. Early experiments have shown that histone inhibits RNA synthesis and RNA stimulates it. If their functions were indeed antagonis-

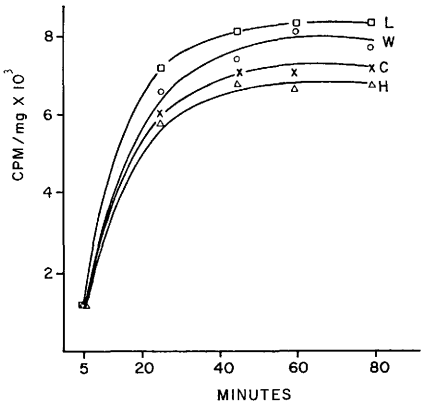

FIG. 5. The effect of whole liver (W), high (H) and low (L) molecular weight RNAs (10 *A*/ml) on the incorporation of ^{3}H-uridine into ^{3}H-RNA.

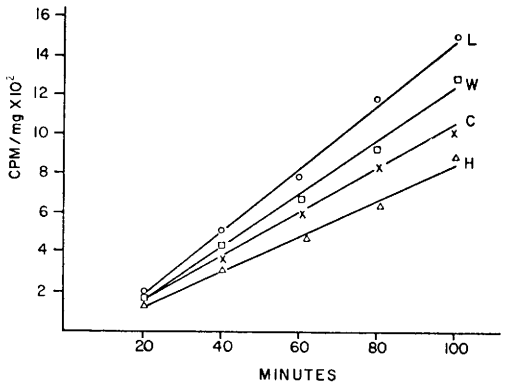

FIG. 6. The effect of whole liver (W), high (H) and (L) molecular weight RNAs (10 A/ml) on the incorporation of ^{3}H-uridine triphosphate into ^{3}H-RNA.

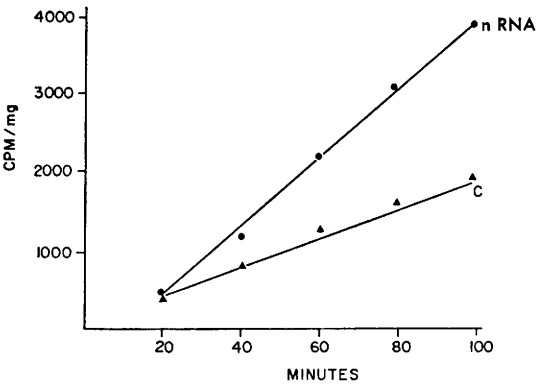

FIG. 7. The effect of nucleolar RNA (10 A/ml) on the incorporation of ^{3}H-uridine triphosphate into ^{3}H-RNA.

tic, the histone-inhibited RNA synthesis would be lifted by addition of RNA and conversely the RNA-stimulated synthesis lowered by histone. This was demonstrated by pretreating ascites cells with histone or RNA for 2 hr in room temperature and then incubating them at 37° in the presence of RNA or histone, respectively. It can readily be seen in Fig. 9 that RNA has brought up the RNA synthesis of the histone-treated cells to a level between the RNA-treated and control and histone reduced the RNA-stimulated synthesis to a level slightly above the histone series. It should be mentioned that the RNA or histone-pretreated cells were metabolically the same in incubation mixture with or without respective RNA or histone. Viability of cells in all series was in the range of 85–95%.

In a dozen or so experiments, histone was inhibitory. Occasionally one of the RNA preparations might not show an appreciable effect on the RNA synthesis. When this RNA was supplied to the histone-pretreated cells, however, an increased RNA synthesis was observed (Fig. 10). The curve labeled RNAse shows the result of the experiment using RNAse-digested RNA. The inhibition, we believe, was due to RNA digestion and partly to the added enzyme which was not removed from the incubation mixture.

Discussion. RNA stimulated the incorporation of ^{3}H-uridine or ^{3}H-uridine triphosphate into acid-insoluble material (^{3}H-RNA). This capability was destroyed by treatment with pancreatic RNAse. The increment of ^{3}H-uridine incorporation was S-shaped and that of ^{3}H-uridine triphosphate was linear. This difference suggests that the action of RNA is multiple. It might activate the enzyme (RNA polymerase), induce new enzyme synthesis and/or function in some other ways associated with RNA synthesis.

Autoradiographic study disclosed that the nuclear uptake of ^{125}I-histone, *i.e.*, nuclear accumulation of silver grains, was predominant. The fact that histone inhibited RNA synthesis in the treated cells provided evidence in support of its action on nuclear biosynthetic apparatus, the chromatin complex (15, 16). From the experiments dealing with functional antagonism between RNA

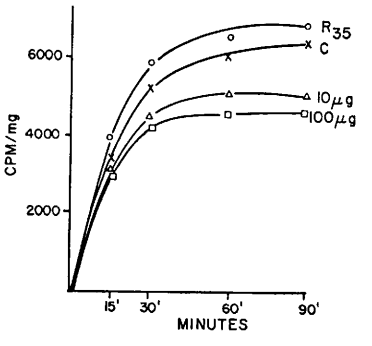

FIG. 8. Comparative effect of liver RNA (35 A/ml) and poly I-C (10 and 100 μg/ml) on the incorporation of ^{3}H-uridine into ^{3}H-RNA.

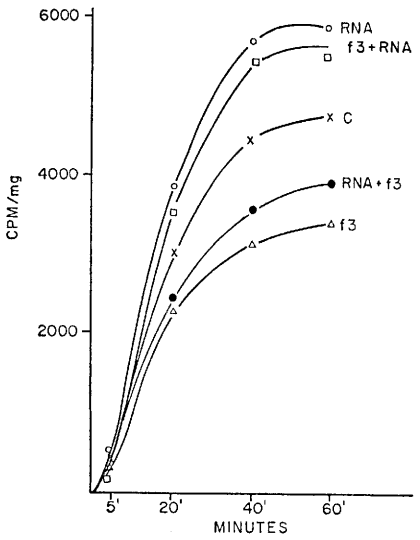

FIG. 9. The effect of RNA on the histone pretreated ascites cells (f3 + RNA) and, conversely, the histone effect on the RNA pretreated cells (RNA + f3) (^{3}H-uridine as precursor). Note the effect of RNA (10 *A*/ml) and histone (100 μg/ml) by comparing the RNA and histone (f3) series, respectively, with the control (C).

and histone, it appeared that the RNA action was mediated through the release (18) or incapacitation (19) of histone.

Actinomycin is known to inhibit DNA-dependent RNA synthesis. The sensitivity of the RNA-stimulated RNA synthesis to actinomycin suggests the involvement of RNA in regulation of nuclear function. This is supported by: (a) exogenous RNA entered the nucleus of the RNA-treated cells (3, 4, 20), (b) RNA synthesis of the chromatin isolated from thymocytes was stimulated by addition of RNA (18), and (c) puromycin inhibited protein synthesis of the RNA-treated cells, but not RNA synthesis. It follows that the action of RNA would not be mediated through product(s) of protein synthesis, and instead by some complex formation with nuclear biosynthetic apparatus.

Histone uptake was studied *in vitro* cultured cells using fluorescent antibody. Its distribution was limited to cytoplasm at pH 7 and in nucleus at pH 4 and below (21). This does not contradict to our finding because ascites cells we used were taken from medium with pH 7.3–7.6. Furthermore, we noted also more histone in cytoplasm than in nucleus at pH 6.9.

The minimal amount of liver RNA used was 500 μg (10 *A*)/ml and the poly I-C was 10 μg. This difference, we believe, was due to the heterogeneity of liver RNA. The pertinent question is which component of liver RNA is most potent? How can it be fractionated and characterized? Is the mode of action different from the nucleolar RNA? Attempts to answer these and other related problems are in progress.

Summary. Functional interaction between histone and RNA was studied using Nelson mouse ascites cells. Liver RNA was capable of stimulating RNA synthesis and arginine-rich histone inhibited it. The potency of low molecular weight fraction was superior to the high and whole RNAs, but inferior to the nucleolar RNA. Poly I-C was unable to lift the RNA synthesis to a level above the controls.

Intracellular distribution of ^{125}I-histone was traced by high resolution autoradiography. The predominant nuclear uptake of histone coupled with early work on RNA uptake provided evidence for the *in situ* action of histone and RNA. The RNA-stimulated RNA synthesis was sensitive to actinomycin

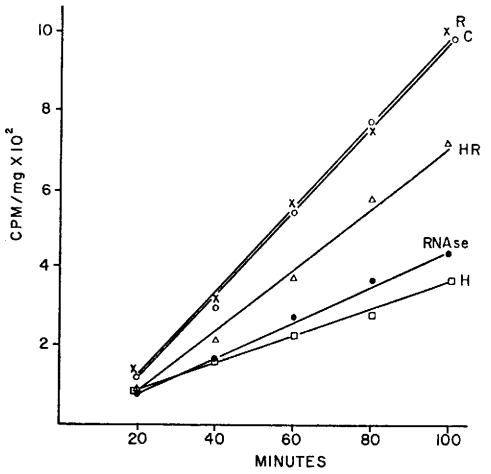

FIG. 10. The response of the histone pretreated cells (H) to RNA (HR) and RNAse-digested RNA (RNAse). The concentration of histone and RNA is same as Fig. 9 (^{3}H-uridine triphosphate as precursor).

D. From studies on the action of RNA versus histone, it was concluded that the action of RNA was mediated by arginine-rich histone.

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