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*ON THE ROLE OF HISTONES IN REGULATING RIBONUCLEIC ACID SYNTHESIS IN THE CELL NUCLEUS**

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The experiments to be described in this paper are concerned with the role of histones in nuclear function and chromosome structure. They deal with RNA synthesis in isolated nuclei or in chromatin strands, with the effects of different histones on this and other biosynthetic processes, and with the enhancement of RNA synthesis in histone-depleted nuclei.

We have shown previously that the addition of histones to isolated nuclei, or to nuclear ribosomes, inhibits or represses a number of their biosynthetic activities.[1-9] For example, one can observe a decrease in the rate of incorporation of C^{14}-labeled amino acids into protein[1-5] or of C^{14}-adenosine into RNA[5, 7-9] when isolated thymus nuclei are given a supplement of basic proteins such as histones, protamines or polylysine. The inhibition observed is due in part to the fact that added histones can inhibit nuclear ATP synthesis[9] and in this way prevent the ATP-dependent "activation" of amino acids needed for protein synthesis[10, 11] and also diminish the kinase activities and ATP "pool" required for RNA synthesis.[12, 13, 6] Such indirect effects are not surprising, considering that histones are known to have a very broad spectrum of inhibitory actions due to their ability to form complexes with many

enzymes (e.g., cytochrome oxidase[14]) or with their substrates or necessary cofactors. Indeed, the toxicity of the histones has been demonstrated in intact, living cells.[15, 16]

Because of the easy combination between the positively-charged histones and negatively-charged polymers, DNA in particular, one would also expect the addition of histones to influence the enzymatic synthesis of polynucleotides by complexing with the necessary DNA "primers."[6, 13, 17, 18] In this connection, the inhibition of RNA synthesis in thymus nuclei by thymus histones has already been described,[5-7] and recent elegant experiments by Huang and Bonner have shown that DNA-histone complexes fail to act as "primers" for RNA synthesis in the presence of an RNA polymerase from pea seedling nuclei.[19]

Is the repression of RNA synthesis *in vitro* by added histones an indication of their true biological function, or is it simply another instance of their general capacity to combine with and inhibit complex enzyme systems?

The experiments to be described below deal with this question. They offer some new and direct indications that histones do play a role in the regulation of nuclear RNA synthesis and that the mechanism of this regulation involves more than a simple inhibition. Two types of evidence are presented; the first compares different histones for their effects on the synthesis of nuclear RNA, DNA, and protein. The second approach is based on the removal of histones from the nucleus and shows: (1) that histone-depleted nuclei have an increased rate of RNA synthesis, and (2) that the newly-formed RNA includes "messenger"-RNAs which differ in composition from the bulk of the RNA in the thymocyte nucleus. The implications of these findings for chromosome function in differentiated cells are discussed.

Materials and Methods.—Isolation of nuclei: Nuclei were isolated from fresh calf thymus tissue after breaking the cells in hypotonic sucrose solution (0.20 *M* sucrose–0.003 *M* $CaCl_2$). The nuclei were then separated in 0.25 *M* sucrose as described elsewhere.[20-22] Most preparations contain fewer than 5 cells per 100 nuclei as judged by electron microscopy.

Preparation of histones: The *total histones* of thymus nuclei were prepared by acid extraction of nuclei previously washed with 0.1 *M* "tris" buffer (pH 7.6) to remove other soluble proteins and nuclear ribosomes.[23] The 0.1 *N* HCl extract was dialyzed briefly against 0.01 *N* HCl, then against water. About 32% of the nuclear dry weight is extractable in this way.

The *lysine-rich histones* were prepared by the method of Johns and Butler,[24] using isolated nuclei rather than whole tissue. The lysine-rich Histone Fraction II was isolated by the method of Daly and Mirsky.[25]

The *arginine-rich histones* were also prepared by the method of Johns and Butler,[24] starting with the isolated nuclei. The arginine-rich Histone Fraction I was isolated by isoelectric precipitation at pH 10.6.[25] Some arginine-rich and lysine-rich histones were prepared by chromatography on carboxymethyl-cellulose.[26]

All the above fractions were tested for their effects on nuclear synthetic reactions.

Effects of Added Histones on Synthetic Processes in the Nucleus.—Histone inhibition of RNA synthesis: The synthesis of ribonucleic acid in isolated calf thymus nuclei has been followed using adenine-8-C^{14}, adenosine-8-C^{14}, guanosine-8-C^{14}, orotic acid-6-C^{14}, and P^{32}-orthophosphate. The incorporation of these labeled compounds into nuclear RNA was allowed to proceed for 30 or 60 mins at 37° in the incubation medium described previously.[12, 22] Over 90 per cent of the counts incorporated can be removed by ribonuclease digestion,[12] or isolated in the appropriate nucleotides after KOH digestion and chromatography.[27, 28]

When histones are added to the incubation medium the synthesis of RNA is inhibited, the degree of inhibition depending on the type of histone and its concen-

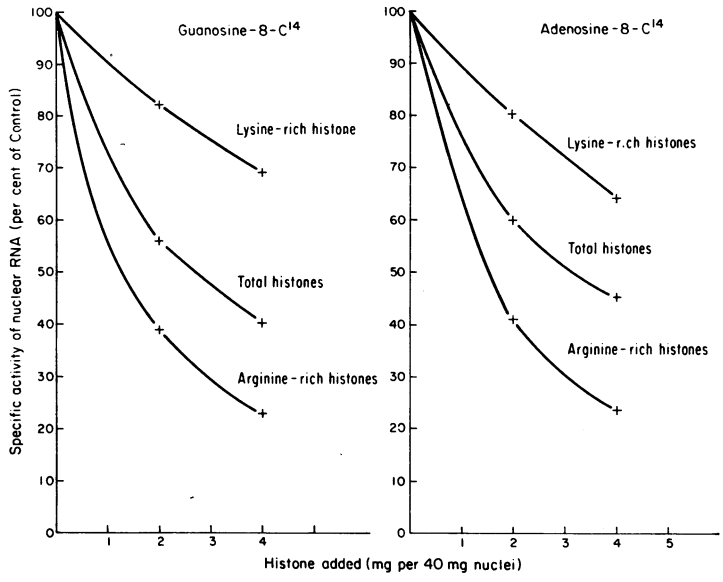

FIG. 1.—The effects of adding different histones on RNA synthesis in isolated thymus nuclei. The amount of histone added is plotted against the relative specific activity of the total nuclear RNA.

tration. The arginine-rich and lysine-rich fractions behave very differently in this regard. Figure 1 summarizes some results obtained using C^{14}-guanosine and C^{14}-adenosine as RNA precursors. It can be seen that the arginine-rich histones are strongly inhibitory while the lysine-rich fractions are comparatively ineffectual. The curve for inhibition by the total histone fraction, as expected, lies between these two extremes. Similar results are shown for orotic acid and adenine incorporation in Table 1. It is of interest that all preparations of lysine-rich histones tested were relatively weak inhibitors of nuclear RNA synthesis, although polylysine itself is a good inhibitor (Table 1).

Because histones also inhibit the "transport" of RNA precursors into nuclear "pools,"[8] other tests were carried out to show that the observed inhibition is not due to this effect, and it was found that the utilization of C^{14}-adenosine already in the nuclear "pool" was strongly inhibited by arginine-rich histones.

Histones also influence nuclear RNA synthesis by affecting nuclear energy metabolism. This aspect of histone action is discussed in detail elsewhere,[9, 20] but it should be noted here that the arginine-rich histones are more inhibitory than are the lysine-rich. The inhibition of nuclear ATP synthesis by added histones has many consequences, since it restricts the energy supply needed for the synthesis of RNA, DNA, and protein.

Effects of added histones on nuclear protein synthesis: It has been shown that histones inhibit amino acid incorporation by isolated nuclei[1, 3, 5] and by nuclear ribosomes.[4] A comparison of the different fractions again shows that the lysine-rich histones are comparatively ineffectual, while the arginine-rich histones are strong inhibitors of amino acid uptake.[1] The extent of inhibition is usually as great or greater than the corresponding repression of nuclear RNA synthesis.

Because alanine-1-C^{14} incorporation depends so largely on its "transport" into

TABLE 1

EFFECTS OF DIFFERENT HISTONES AND POLYLYSINE ON RNA SYNTHESIS IN THYMUS NUCLEI

Histone added	mg	Specific Activity of Nuclear RNA after Labeling with: Adenosine-8-C^{14} cpm/mg	% inhibition	Guanosine-8-C^{14} cpm/mg	% inhibition	Orotic acid-6-C^{14} cpm/mg	% inhibition	Adenine-8-C^{14} cpm/mg	% inhibition
Control—no histone	0	1295	...	2465	...	1900	...	967	...
Total histone	1	1049	19.0	1738	29.5	...	...	...	...
"	2	725	44.0	1380	44.0	...	...	...	...
"	4	531	59.0	998	59.5	...	...	...	...
Lysine-rich fraction*	1	1153	11.0	2206	10.5	...	...	916	5.3
"	2	1036	20.0	2021	18.0	1880	1.1	889	8.1
"	4	829	36.0	1700	31.0	1925	0	867	10.3
Lysine-rich fraction†	4	1210	6.6	...	...	...	...	...	...
Polylysine	4	715	44.8	...	...	...	...	...	...
Arginine-rich fraction*	1	803	38	...	...	...	...	...	...
"	2	505	61	...	...	1550	18.4	796	17.7
"	4	298	77	...	...	1390	26.8	542	44.0
Arginine-rich fraction†	4	540	58.3	567	77.0	...	...	...	...

* Prepared by the method of Johns and Butler.[24]
† Prepared by the method of Daly and Mirsky.[25]

nuclear "pools"[8] (a reaction inhibitable by histones[8]), some experiments were carried out in which the amino acid was admitted to the "pool" before adding the histone supplement: alanine incorporation into protein was still inhibited.

Effects of histones on thymidine incorporation into DNA: Isolated thymus nuclei incorporate C^{14}-thymidine into DNA.[31, 6] The addition of the arginine-rich Histone I Fraction (1 mg/ml) reduced uptake from 16 c.p.m. per mg DNA in controls to 5.7 c.p.m. per mg DNA in histone-treated nuclei.

Some effects of removing histones from isolated nuclei—Method: Because the addition of histone supplements to nuclei produces such a wide range of inhibitions, it was decided to approach the problem in a new way—by removing the histones selectively and testing for changes in function or activity of the histone-depleted nuclei. One procedure we have used makes use of trypsin because of its known preferential hydrolysis of peptide bonds involving arginine and lysine. When trypsin is added to nuclear suspensions (at levels of 0.5 mg per ml; 1 mg enzyme per 40 mg nuclei), much of the histone is hydrolyzed and released. The extent of the hydrolysis can be followed roughly by Millon analyses[32] of the acid-soluble proteins which remain in the trypsin-treated nuclei. About 70 per cent of the total histone is removed in 30 min at 37°, while the losses of nonhistone protein are comparatively small.

Enhancement of RNA synthesis: This treatment results in a marked stimulation of nuclear RNA synthesis. Some results are summarized in Table 2, which shows that the uptake of orotic acid, adenine, guanosine, and P^{32}-orthophosphate are all much higher in histone-depleted nuclei than in the corresponding "controls." Stimulations ranging from 200–400 per cent are the rule. The increased radioactivity has been definitely localized in the nuclear RNA, which was extracted in hot 10 per cent NaCl, precipitated with ethanol, hydrolyzed in KOH and chromatographed on Dowex-1 to separate the component nucleotides (Table 2).

TABLE 2

STIMULATION OF RNA SYNTHESIS IN ISOLATED THYMUS NUCLEI FOLLOWING HISTONE REMOVAL BY TRYPSIN

Conditions of experiment	Precursor added	Specific Activity of RNA Nucleotides: Total nucleotides cpm/mg	Adenylic acid cpm/μmole	Cytidylic acid cpm/μmole
Nuclei—alone	Orotic-6-C^{14} acid	74.1	...	...
" + 0.5 mg trypsin	" "	157.	...	...
" + 1.0 mg trypsin	" "	330.	...	...
Nuclei—alone	Adenine-8-C^{14}	330.	97.	...
" + 1.0 mg trypsin	" "	887.	256.	...
Nuclei—alone	Guanosine-8-C^{14}	663.	...	...
" + 1.0 mg trypsin	" "	1390.	...	...
Nuclei—alone	P^{32}-orthophosphate	1388.	9,380.	6,400.
" + 1.0 mg trypsin	" "	3330.	26,070.	18,860.

Because trypsin digestion not only releases histones but also hydrolyzes some other proteins of the nucleus, inhibitory effects are seen in other synthetic systems. Amino acid incorporation usually drops by about 60 per cent.

Trypsin digestion disrupts nuclear structure (see below). To avoid this structural complication, the enzyme action was moderated by the addition of the crystalline trypsin inhibitor from soy bean.[33] When 0.8 mg of inhibitor was added per mg of enzyme, the nuclei remained intact (Fig. 3, *B*) while histone digestion proceeded due to the trypsin excess. Nuclear RNA synthesis was greatly enhanced, as shown in Figure 2, which plots the specific activity of nuclear RNA against the time of incubation. It can be seen that trypsin-treated nuclei (curve 3) are far more active in incorporating C^{14}-adenine and guanosine than the corresponding

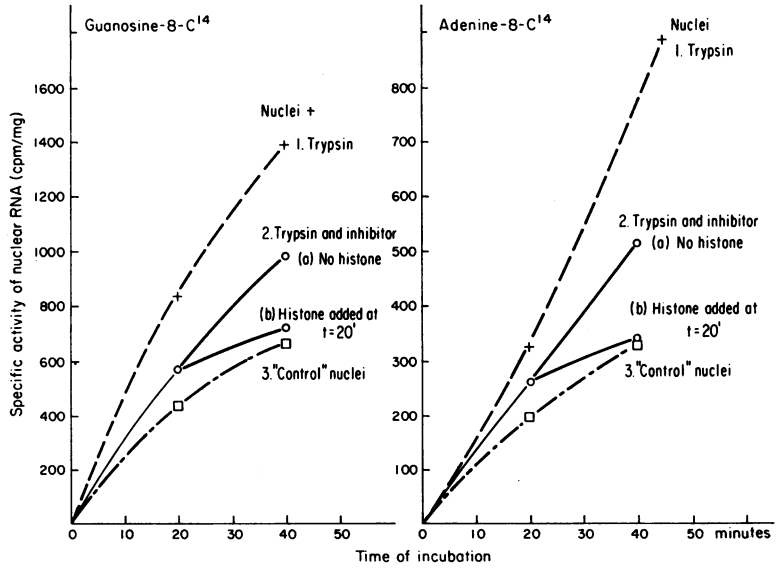

FIG. 2.—The effect of removing histones on nuclear RNA synthesis. The incorporation of guanosine-8-C^{14} and adenine-8-C^{14} is plotted as a function of the time of incubation at 37°. The lower curve (curve 3) shows the time course of C^{14} uptake in control nuclei. The upper curve (curve 1) shows the uptake in nuclei which were incubated with trypsin to remove 70% of the total histones. Curve 2 (a) is for nuclei treated with trypsin + soy inhibitor to moderate the enzyme action. Curve 2 (b) shows that the addition of 4 mg of histone at t = 20′ again suppresses nuclear RNA synthesis.

"controls" (curve 1). Nuclei treated with trypsin plus the soy inhibitor show about a 70 per cent increase in their uptake of these RNA precursors (curve 2*a*). The figure also shows that when histone is added back to the histone-depleted nuclei at 20 min, the synthesis of RNA is again inhibited (curve 2*b*).

Increased synthesis of "messenger"-RNAs in histone-depleted nuclei: We have shown previously that thymus nuclei contain[34] and actively synthesize[6] an RNA fraction of base composition similar to that of thymus DNA. The isolation of this "messenger"-RNA fraction has been carried out following the procedure of Sibatani *et al.*[34] after a 30 min incubation in the presence of P^{32}-labeled orthophosphate. In "control" nuclei the specific activity of the "messenger"-RNA fraction was 29,960 cpm per mg RNA-P; in trypsin-treated nuclei the corresponding figure was 90,150 cpm per mg RNA-P, an increase of over 300 per cent.

Some preliminary data on the distribution of P^{32} in the RNA nucleotides suggests that the composition of the newly synthesized RNA differs from that normally being made in the thymus nucleus. Increased P^{32} uptakes into guanylic and uridylic acids have been observed, but it remains to be proved that the removal of histones has activated previously "repressed" DNA primers of different nucleotide composition.

Structural effects of trypsin treatment: When nuclei are incubated in the presence of trypsin alone, as in the above experiments, there are profound changes in structure. Macroscopically, the suspension forms a thick gel. Electron microscopy of the gel shows that it consists of thin strands of chromatin, of varying diameters and degrees of aggregation. The smallest strands are about 50 Å in diameter (Fig. 3, *C* and *D*). If the incubation is carried out using trypsin plus trypsin inhibitor, gel formation does not occur, though much of the histone is digested. The nuclei after digestion appear normal, as judged by light and electron microscopy. (Compare Fig. 3, *B* with the untreated nuclei shown in Fig. 3, *A*.) It is a remarkable fact that nuclear structure can be disrupted by trypsin and still permit RNA synthesis to continue, and at a higher rate.

Discussion.—The evidence presented above indicates that histones can inhibit or repress a large number of nuclear biosynthetic activities, including RNA synthesis. The mechanism of the inhibition is complex and may involve several steps in the utilization of a precursor, beginning with transport and including ATP-dependent kinase reactions as well as the final polymerase reaction. The arginine-rich histones are strong inhibitors while the lysine-rich fractions are weakly inhibitory. These findings may be tentatively combined with earlier observations that most of the DNA in the thymus nucleus, or in other highly differentiated cell types, is "inactive" or "repressed" and can be removed, while the remaining DNA governs RNA synthesis.[6] One could suggest a model of chromosome structure in which most of the DNA was bound to histone fractions, such as the arginine-rich histones, which inhibit the synthesis of "messenger"-RNAs. Active genes or "priming" sites would contain the lysine-rich histone fractions or a histone complex which favors DNA utilization as a primer for RNA synthesis. Release of the arginine-rich histones would be expected to result in increased "messenger"-RNA synthesis and lead to the production of previously repressed "messages." The experimental data presented in this paper are in accord with such a model.

Summary.—The addition of thymus histones to isolated thymus nuclei inhibits

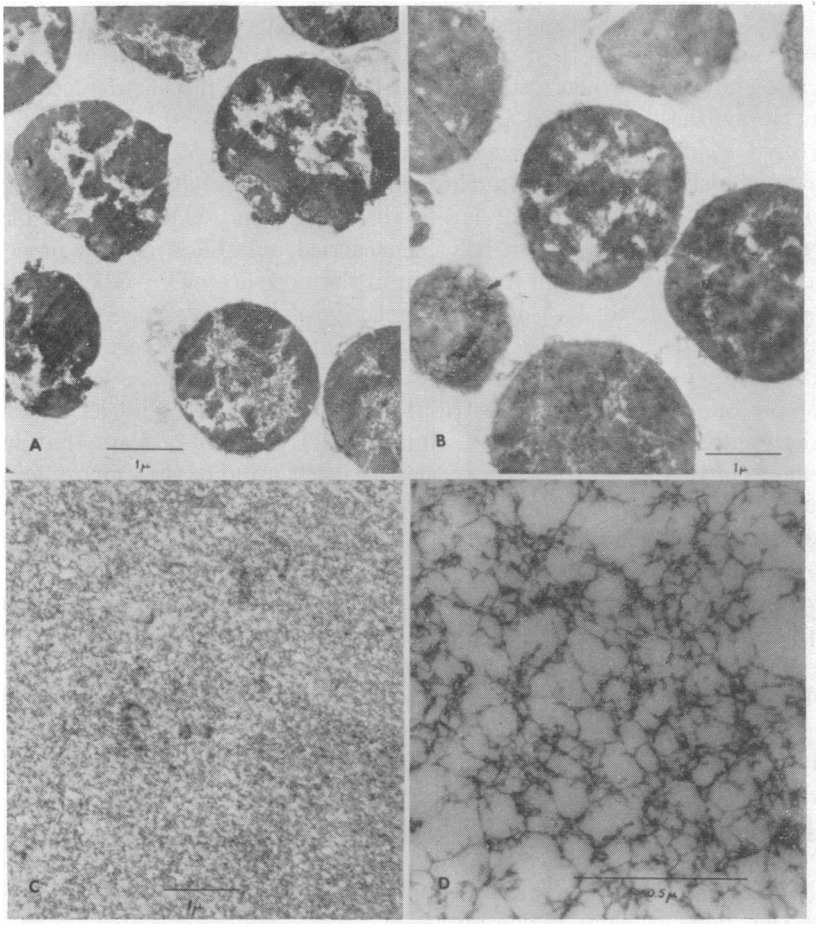

FIG. 3.—Electron micrographs of calf thymus nuclei before and after trypsin digestion.
A: Control nuclei, incubated for 20 min in the absence of trypsin.
B: Nuclei incubated with 1 mg trypsin plus 0.8 mg trypsin inhibitor for 20 minutes at 37°.
C: Gel remaining after trypsin treatment of nuclei.
D: Same as C, at higher magnification.

many biosynthetic reactions, including RNA synthesis. The arginine-rich histones are strong inhibitors; the lysine-rich histones are relatively inert. Selective removal of the histones from the nucleus results in increased rates of RNA synthesis. The newly synthesized RNA occurs in the "messenger"-RNA fraction and may differ in its base composition from the usual "messenger"-RNA complement of the thymus. Some implications for chromosome structure and function are discussed.

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GENETICS: A CORRECTION

The classification *Biochemistry* for the article entitled "Interaction of Genotypes Determining Viability in *Drosophila busckii*," by R. C. Lewontin and Yoshiro Matsuo, which was published in the February issue of these PROCEEDINGS (vol. 49, no. 2, pp. 270–278), should have been *Genetics*.