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*HISTONE, A SUPPRESSOR OF CHROMOSOMAL RNA SYNTHESIS**

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We have previously reported[1, 2] that chromatin isolated from pea embryos possesses the ability to carry out the DNA-dependent synthesis of RNA from the four riboside triphosphates.[3] The present paper concerns the roles in such synthesis of the several components of chromatin. It will be shown that the DNA of pea embryo chromatin is present in at least two forms, namely, as DNA itself and as DNA bound in nucleohistone complex. It will be further shown that DNA fully complexed with histone is inactive in the support of DNA-dependent RNA synthesis.

Materials and Methods.—Pea embryos: Pea seeds (var. Alaska) were germinated in 35-gallon barrels in lots of 25 lb. The seeds were soaked for 5 hr in running water at 20°C and then gently sprayed with water for an additional 35 hr. The embryonic axes, approximately 1 cm in length, were next separated from the cotyledons in a semiautomatic 3-stage disassembly line. Fifty pound dry weight of seeds yield approximately 1 kg fresh weight of embryos.

Preparation of chromatin: The chilled, sterilized (with 100× diluted Clorox) embryos were ground for approximately 1 min in a Blendor with an equal weight of grinding medium (sucrose 0.25 *M*, tris pH 8.0, 0.05 *M*, β-mercaptoethanol, 0.01 *M*, $MgCl_2$, 0.001 *M*) and filtered successively through cheesecloth and miracloth to remove cell wall debris. The filtrate was then centrifuged for 30 min at 4,000 × *g*. Under these circumstances, mitochondria and smaller particles remain in suspension while starch and chromatin sediment. The gelatinous chromatin layer was scraped from the underlying, firm starch layer and washed by successive recentrifugation (10,000 × *g*) in grinding medium (1×), sucrose, 0.25 *M* (2×), and tris, 0.05 *M*, pH 8.0 (2×). β-Mercaptoethanol, 0.01 *M*, was included in all of the above media. The final pellet was resuspended in the tris buffer, 30 ml per kg initial embryos. The yield of such crude chromatin is approximately 0.5 gm per kg embryos; the yield of DNA, 50 mg per kg embryos.

Purification of crude chromatin: The crude chromatin prepared as described above contains *ca.* 95% of the DNA of the embryo but is contaminated by nonchromosomal protein, removable by sucrose gradient centrifugation. This was accomplished by layering 5 ml of crude chromatin suspension on 25 ml of 2 *M* sucrose (0.01 *M* in β-mercaptoethanol). The upper third of the tube was then gently stirred to form a rough gradient and the tubes centrifuged in the SW-25 swinging bucket head at 20 krpm for 3 hr. The resulting pellet of which the major constituents are DNA and histone will be referred to as purified chromatin. Approximately 70% of the DNA of crude chromatin is recovered in the purified chromatin.

Analyses: DNA and RNA were determined principally by the Schmidt-Tannhauser procedure according to Ts'o and Sato.[4] The diphenylamine method of Burton[5] was used for determination of DNA in the presence of much protein. Total protein was determined by the Folin-phenol method as described by Lowry.[6] Histone protein was separated from total protein on the basis of the solubility of the former in 0.2 *N* HCl and determined on the acid extract by the Lowry method. Melting point determinations of DNA and nucleohistone were carried out

with the Beckman DK-2 spectrophotometer equipped with a controlled-temperature cell.

Separation of chromosomal nucleic acid from chromosomal protein: For the resolution of chromatin into its nucleic acid and protein constituents, the chromatin suspension (9.5 ml) was homogenized with neutral 6.6 *M* CsCl (15 ml). The resulting suspension, 4.0 *M* in CsCl and of density 1.40, was next centrifuged in the SW-39 swinging bucket rotor at 35 krpm for 40 hr. The clear gelatinous pellets contain the DNA and a portion of the RNA of the original chromatin. The protein collects at the top of the tube as a skin. Further purification of the nucleic acid pellet could be achieved by a second cycle of such centrifugation.

Preparation of deproteinized DNA: The DNA of pea embryo chromatin was also deproteinized by the Sevag process as modified by Marmur.[7]

Preparation of native chromosomal histone: It has proved difficult for us to recombine DNA with histone prepared by the usual acid extraction without causing aggregation of the DNA. Native, non-acid-extracted histone elicits this behavior to a lesser extent. We have purified native histone from pea chromatin as follows: The protein skin from CsCl fractionation is first thoroughly extracted (2×) with tris (0.05 *M* pH 8.0) to remove nonhistone protein. The histones are then extracted from the residue with 2.5 *M* NaCl. The clear, salt-soluble protein extract is then reprecipitated by dialysis against tris (0.05 *M*, pH 8.0). The precipitated histone is next centrifuged off and redissolved in 2.5 *M* NaCl. The protein so prepared is completely soluble in 0.2 *N* HCl, as histones should be.

Preparation of soluble RNA polymerase: Soluble RNA polymerase was extracted with tris buffer (0.05 *M*, pH 8.0, 0.01 *M* in β-mercaptoethanol) from the protein skin separated from chromatin by centrifugation in 4 *M* CsCl. The soluble RNA polymerase of the initial extract was purified by addition of 0.05 volume of 10% streptomycin solution followed by centrifugation. To the supernatant was added 0.04 volume of 1% protamine sulfate. The precipitate was centrifuged off (10 krpm, 20 min) and extracted with 0.1 *M* ammonium sulfate in tris (0.05 *M*, pH 8.0, and 0.01 *M* in β-mercaptoethanol). This procedure is similar to that of Chamberlin and Berg.[8] The thus purified enzyme still contains some endogenous DNA.

Incubation and washing: Determination of the RNA-synthesizing ability of enzymatic extracts was carried out in a standard reaction mixture containing per 0.5 ml: 0.5 μm each of ATP, GTP, CTP, and UTP, 10 μm of $MgCl_2$, 3 μm β-mercaptoethanol, 50 μm tris buffer (pH 8.0), and the enzyme and DNA samples under investigation.

Either 8-C_{14} ATP or 2-C_{14} CTP (1.0 μC/1.0 μm) was used as label for following synthesis of RNA. Rate of incorporation of the two labeled substrates was close to identical in this system. Incubation was at 37°C. At the end of the incubation period, the reaction was stopped by the addition of 20 volumes of cold 5% TCA. The precipitate was washed two further times with 5% TCA and this followed by an alcohol wash. The washed precipitate was next redissolved in 2 *M* NH_4OH and either dialyzed overnight and the nondialyzable material counted or twice reprecipitated with 5% TCA. The final precipitate, redissolved in NH_4OH, was plated for counting. That the material into which the carbon of 8-C_{14} ATP is incorporated by the present system is in fact RNA has been shown in previous work.[2, 3]

Experimental Results.—Composition of chromatin: Pea embryo chromatin separated by differential centrifugation and purified by sucrose gradient centrifugation as described above, consists of four principal constituents, DNA, RNA, histone and nonhistone protein. The data of Table 1 show that DNA and histone are present in very nearly equal amounts. The ratio of RNA to DNA in chromatin varies from *ca.* 1:3 to *ca.* 1:2, the RNA making up less than 20 per cent of the chromosomal mass. Nonhistone protein, constituting 15–20 per cent of the chromosomal mass, includes, as will be shown below, the chromosomal RNA polymerase.

TABLE 1

COMPOSITION OF PURIFIED PEA EMBRYO CHROMATIN

Component	Mg/100 gm pea embryos	Per cent of total mass
DNA	3.42	31
RNA	1.90	17.5
Histone protein	3.67	33
Nonhistone protein	1.95	18

Pea embryo chromatin as obtained by the present procedures consists principally of aggregates of nucleoprotein strands in which the histone is intimately associated with the DNA. Binding of histone to DNA stabilizes the structure of the latter, as is evidenced by the fact that the melting temperature of chromosomal nucleohistone is higher than that of the corresponding pure DNA. As is shown in Figure 1, the temperature at which half melting has occurred, T_M, is 84° for pea

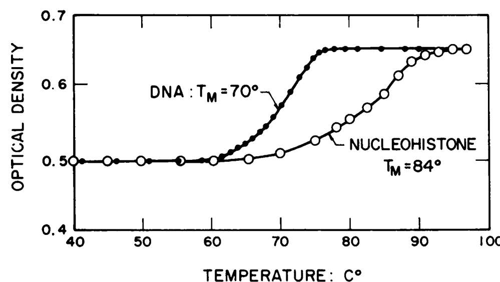

FIG. 1.—Melting profiles of DNA of pea embryo chromatin in the form of (left) deproteinized DNA (Sevag procedure) or (right) native chromosomal nucleoprotein. In both cases, the samples were dispersed in dilute saline citrate, 0.016 *M*.

chromatin nucleohistone under conditions for which the T_M of pea DNA is 70°. A similar observation for thymus nucleohistone has been qualitatively reported by Zubay and Doty.[9]

Purification of chromosomal RNA polymerase: It has been found that elegant separation of the bulk of the chromosomal protein from the bulk of the chromosomal nucleic acid may be achieved by dispersal and centrifugation of chromatin in 4 *M* CsCl. At this ionic strength, histone is dissociated from DNA. At the density of 4 *M* CsCl (1.40), nucleic acid pellets while protein floats, forming a skin. This skin includes all of the chromosomal histone and a portion of the chromosomal nonhistone protein. Chromosomal nucleic acid prepared by two cycles of centrifugation in 4 *M* CsCl possesses the properties summarized in Table 2. The DNA prepared

TABLE 2

PROPERTIES OF CHROMOSOMAL NUCLEIC ACID PREPARED FROM PEA EMBRYO CHROMATIN BY TWO CYCLES OF CENTRIFUGATION IN 4 *M* CSCL

Property	Value
DNA content	90%
RNA content	6%
Protein content	4%
Sedimentation constant of DNA	18 *S*

by the CsCl method, like DNA officially deproteinized by the Sevag process, is molecularly dispersed as particles of sedimentation constant approximately 18 *S* (in standard saline citrate, 0.16 *M*). Such DNA contains bound to it nonetheless *ca.* 4% of protein (approximately 3.5% of the total chromosomal protein). This DNA-bound protein includes a portion of the chromosomal RNA polymerase since the preparation is active in RNA synthesis. The data of Table 3 show that by the CsCl fractionation procedure the specific RNA-synthesizing activity of the nucleic acid-bound RNA polymerase is increased approximately 1,000-fold above that of the initial crude chromatin.

Although a portion of the chromosomal RNA-polymerase remains associated with DNA during high salt concentration-induced disaggregation of chromatin, a further portion of the enzyme is released and accompanies the histone. The thus solubil-

TABLE 3

PURIFICATION OF RNA POLYMERASE ASSOCIATED WITH PEA EMBRYO CHROMATIN

Preparation	RNA-synthesizing activity (μμm nucleotides incorporated into RNA/10 min/mg protein)
1. Initial chromatin	117
2. Purified chromatin*	1,100
3. Enzyme associated with DNA†	118,000
4. Solubilized enzyme‡	15,800

* Nonchromosomal protein removed by sucrose gradient centrifugation. † DNA-enzyme complex, 2× pelleted in 4 *M* CsCl. ‡ Extracted and purified from protein released from chromatin in presence of 4 *M* CsCl. Tested in presence of 50 μg added pea DNA.

ized enzyme, purified by streptomycin and protamine precipitation as described under *Methods*, contains some endogenous DNA but is nevertheless capable of carrying out RNA synthesis in response to added DNA (Table 4). In this function, pea DNA may be replaced by DNA of other origin as thymus or phage T-7. For some presently inexplicable reason, pea RNA polymerase actually prefers and is more active in response to such foreign DNA.

TABLE 4

UTILIZATION OF VARIOUS KINDS OF DNA IN THE SUPPORT OF RNA SYNTHESIS BY RNA POLYMERASE OF PEA EMBRYO CHROMATIN

Source of DNA	RNA synthesized/10 min. μμm nucleotide incorporated
None	384
Pea	960
T7	1,680
Thymus	2,100

Fifty μg DNA added per 0.5 ml standard reaction mixture (*Methods*) and in presence of 1 mg crude enzyme.

Role of histone: Chromatin as obtained from pea embryos is of course rich in DNA. This DNA as it occurs in chromatin is, however, remarkably inefficient in the support of DNA-dependent RNA synthesis. DNA as it is present in chromatin is less effective in supporting RNA synthesis than an equal amount of pure DNA in the presence of soluble RNA polymerase. It appears therefore that there is in the chromosome some factor which renders the chromosomal DNA not fully effective in RNA synthesis. That the factor inhibitory to RNA synthesis is the chromosomal histone is shown by the data of Table 5. The two reaction mixtures of Table 5 concern the same initial amount of chromatin. From one, protein and nucleic acid

TABLE 5

INCREASE IN THE RNA-SYNTHESIZING ACTIVITY OF CHROMATIN BY REMOVAL OF HISTONE

System	RNA synthesized/10 mg original crude chromatin μμm nucleotide incorporated/10 min
Crude chromatin*	400
Solubilized chromatin, histone removed†	2,090

* Histone:DNA ratio = 1:1.1. † Solubilized by 4 *M* CsCl. Nonhistone protein and nucleic acid recombined.

were separated by centrifugation in 4 *M* CsCl. The resultant protein skin was then extracted with tris buffer (0.05 *M*, pH 8.0) and the extracted nonhistone protein recombined with the nucleic acid from which it had previously been removed. The two reaction mixtures represent then chromatin with and without histone. Re-

moval of histone results in an increase in the RNA-synthesizing activity of the preparation by approximately fivefold.

Reconstitution of nucleohistone: To further elucidate the role of histone in DNA-dependent RNA synthesis, nucleohistone has been reconstituted from its purified components and the reconstituted material tested for its ability to replace DNA in the DNA-dependent synthesis of RNA. For this purpose, native histone prepared as described under *Methods* by repeated solubilization at high ionic strength (2.5 NaCl, pH 8.0) and precipitation at low ionic strength (0.05 *M* tris, pH 8.0) was used. DNA deproteinized by the Sevag procedure was used as the nucleic acid. For reconstitution, histone and DNA were mixed in a mass ratio of approximately 2:1 in solution of high ionic strength (1.0–2.5 NaCl) and slowly brought to low ionic strength (0.05 *M* tris, pH 8.0) by gradient dialysis over a period of several hours.

At the end of this period, any unbound (and hence precipitated) histone and any aggregates of DNA and histone were centrifuged off at 10,000 × *g*. The resultant nucleohistone is soluble and yields a clear solution. That DNA is bound to histone in the reconstituted nucleohistone preparations is indicated by three facts, namely (1) the presence of DNA increases the solubility of histone in low ionic strength solution, (2) the sedimentation constant of the reconstituted nucleohistone is greater than that of the DNA, and (3) the melting temperature of the DNA is increased by the presence of histone as is shown in Figure 2. The T_M of the preparation of Figure 2, 79°C, is not as high as that of native nucleohistone at the same ionic strength (84°C) but is nonetheless substantially above that of free DNA (70°C).

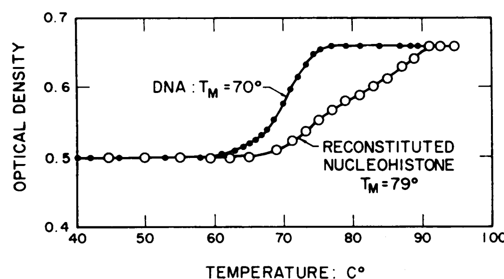

FIG. 2.—Melting profiles of DNA of pea embryo chromatin in the form of (left) deproteinized DNA or (right) nucleohistone reconstituted from pea chromatin DNA and pea chromatin histone in the proportion of 1:1.04. Both dispersed in dilute saline citrate, 0.016 *M*.

Inactivity of nucleohistone in support of RNA synthesis: The data of Table 6 indicate that, in the support of DNA-dependent RNA synthesis, the reconstituted nucleohistone is inactive or nearly so. That the nucleohistone does not inactivate

TABLE 6

THE EFFECT OF HISTONE ON DNA AS PRIMER IN RNA SYNTHESIS

System	RNA synthesized/10 min. μμm nucleotide incorporated
Enzyme alone	720
Enzyme + DNA	1,440
Enzyme + reconstituted deoxynucleohistone (Histone:DNA ratio 1.04:1)	576
Enzyme + DNA + histone	0

50 μg DNA (either deproteinized by the Sevag process or as nucleohistone) added in 0.5 ml standard reaction mixture (*Methods*) in presence of 940 μg crude enzyme. The fourth reaction mixture contained in addition to 50 μg DNA, 50 μg histone.

or otherwise influence the RNA polymerase is shown by the fact that free DNA, in a reaction mixture containing nucleohistone, is fully effective in support of the RNA polymerase reaction. DNA is not irreversibly influenced by combination with histone. Thus, reconstituted nucleohistone was again separated into histone and DNA by centrifugation in 4 M CsCl. The recovered DNA exhibited a T_M of 70° (in 0.016 M salt) and was as effective in the support of DNA-dependent RNA synthesis as the original deproteinized (Sevag) DNA.

In further experiments, of the type summarized in Figure 3, purified histone was

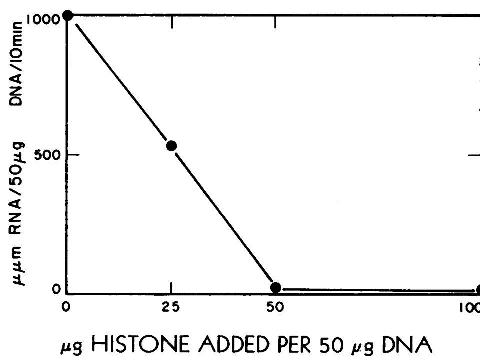

FIG. 3.—Influence of added pea chromatin histone upon DNA-dependent RNA synthesis. Fifty μg DNA per 0.5 ml standard reaction mixture (*Methods*) containing crude enzyme (1.04 mg) plus histone as indicated.

added to a complete DNA-containing reaction mixture immediately before the addition of RNA polymerase. Under these conditions, also, the presence of histone inhibits the RNA-synthesizing ability of the system. The extent of the inhibition depends upon the ratio of added histone to added DNA and becomes complete or nearly so when this ratio is 1 or greater, as is apparent in Figure 3. That such added histone also suppresses the endogenous DNA contained in the RNA polymerase preparation is shown in Table 6.

The inhibition of DNA-dependent RNA synthesis by histone is not due to any obvious enzymatic activity of the material. Thus, our histone preparations are devoid of phosphatase activity as well as of RNAase activity. The fact that native DNA may be recovered from reconstituted nucleohistone shows that the histone has no important DNAase activity.

Discussion.—The facts presented above indicate that chromatin consists, so far as RNA synthesis is concerned, of three relevant components, DNA, RNA polymerase, and histone. The RNA polymerase possesses the power to catalyze RNA synthesis in the presence of DNA. The function of the histone is to bind to DNA. The evidence presented above indicates that DNA fully complexed with histone is inactive in the support of DNA-dependent RNA synthesis. Since chromatin as obtained from the pea embryo possesses some ability to conduct DNA-dependent RNA synthesis, it appears probable that a portion of the DNA of pea chromatin is not fully occupied by histone and that it is this fraction of the DNA which is active in RNA synthesis. In the sense that it suppresses the ability of a DNA molecule to participate in DNA-dependent RNA synthesis, histone is a suppressor of genetic activity. That histone might act as a regulator of genetic activity has been suggested as a speculation by Stedman and Stedman in 1950.[10]

Chromosomal histone is known to consist of a mixture of proteins of varying amino acid composition[11] and sequence.[12] The question of whether there is speci-

ficity of any kind involved in the determination of what kind of DNA binds with what kind of histone is an interesting question but is not considered in this paper.

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*THE ROLE OF DEOXYRIBONUCLEIC ACID IN RIBONUCLEIC ACID SYNTHESIS, III. THE INHIBITION OF THE ENZYMATIC SYNTHESIS OF RIBONUCLEIC ACID AND DEOXYRIBONUCLEIC ACID BY ACTINOMYCIN D AND PROFLAVIN**

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Actinomycin D is one of a number of polypeptide antibiotics isolated in Waksman's laboratory.[1, 2] Bacteriostatic effects, particularly on gram positive bacteria, and antitumor activity have been attributed to this compound.[2, 3] Kirk[4] has demonstrated that the addition of actinomycin D (0.2 to 0.5 μM) to exponentially growing cultures of *Staphylococcus aureus* stops RNA synthesis immediately. This effect is rapidly followed by an inhibition of protein synthesis, and later by a partial inhibition of DNA synthesis. The action of this compound is not related directly to energy production since both respiration and glycolysis of inhibited cells are unaffected by concentrations up to 0.1 mM.[4] Kirk also demonstrated that the combination of DNA and actinomycin D results in a spectral change of the latter compound. These observations suggest the formation of a complex between these two compounds since Kawamata and Imanishi[5] found no interaction of actinomycin and RNA and the reaction appears to be relatively specific for DNA. Although Rauen *et al.*[6] have reported complex formation between actinomycin and RNA, 100 times more RNA than DNA is required.

Two recent reports have suggested that actinomycin acts by inhibiting the synthesis of "messenger" RNA. Nakata *et al.*[7] have shown that reproduction of T2