Polynucleotides Associated with Histone Preparations

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(Received 14 August 1972)

Histone fractions prepared from various sources by acid extraction or by salt dissociation contained small quantities of ribo- and deoxyribo-nucleotides. These nucleotides were small and heterogeneous and were efficiently removed from histone preparations by ion-exchange chromatography. They probably represent contaminants, rather than uniquely complexed nucleotides, of all the histone preparations.

Histones are very basic nuclear proteins, which are usually prepared by extraction of saline-washed nuclei with dilute acids or, sometimes, by dissociation of the nucleoprotein with salt. They are heterogeneous and may be fractionated by several methods to give a small number of major groups. Within some of these groups further heterogeneity can be demonstrated (for review see Stellwagen & Cole, 1969). Nucleated erythrocytes contain a unique histone (fraction V) (Neelin, 1964), which can be resolved into two very similar components (Va and Vb) by chromatography (Greenaway & Murray, 1971). Since RNA-histone complexes have been described (Huang & Bonner, 1965), the possibility that subfractions Va and Vb differed in their associated nucleotides was examined. Nucleotides were not detected in the two chromatographic subfractions but the method showed the presence of appreciable quantities of nucleotides in the extract of histone fraction V before chromatography. Both ribonucleotides and deoxyribonucleotides were present. The analysis was then extended to several other histone preparations and fractions from various sources.

An RNA species first observed in histone preparations from pea cotyledons (Huang & Bonner, 1965) and later found in rat liver and calf thymus (Benjamin *et al.*, 1966; Shih & Bonner, 1969) was believed to be uniquely bound to nuclear proteins. It was small, heterogeneous and of characteristic composition, but was subject to variable, uncontrolled losses during purification. Heyden & Zachau (1971) have since described experiments showing that the RNA bound to nuclear proteins from calf thymus glands was, for the most part, derived from tRNA. It has been shown also by Artman & Roth (1971), Hill *et al.* (1971) and Szeszák & Pihl (1972) that RNA associated with the chromosomal proteins of various chicken tissues, sea urchin embryos and rat liver comprises more than one type of RNA, the majority of which is not

covalently attached to protein. In the present paper we describe some studies on the properties of nucleotides associated with various histone preparations. A highly sensitive procedure was selected for this investigation. This involved phosphorylation of nuclease digests of the histone subfractions by means of the polynucleotide kinase reaction (Richardson, 1965; Novogrodsky & Hurwitz, 1965, 1966), followed by nucleotide 'mapping' of the labelled nucleotides by ionophoresis on ion-exchange papers.

Materials and Methods

Materials

Pooled chicken blood was generously given by J. Sainsbury Ltd., Bury St. Edmunds, Suffolk, and by D. B. Marshall Ltd., Newbridge, Midlothian. Pooled turkey (*Meleagris gallopavo*) blood was generously given by Fenton Barns Ltd., nr. Drem, East Lothian. Erythrocyte histone fractions were prepared by selective acid extraction (Murray *et al.*, 1968) and by salt dissociation (Murray *et al.*, 1970) of saline-washed nuclei. Frozen rabbit thymus glands were obtained from Pel-Freeze Inc., Rogers, Ark., U.S.A., and histones were prepared by acid extraction (Murray, 1966). Preparations of yeast histones and frog (*Rana pipiens*) erythrocyte histones were generously donated by Dr. J. Duffus (Duffus, 1971) and Dr. K. W. Jones. Calf thymus histone fractions were those described by Murray (1966, 1969), and the histone fractions prepared by chromatography on Amberlite CG-50 were those described by Murray (1966) and by Greenaway & Murray (1971). ³H-labelled DNA was prepared from bacteriophage λ (Murray, 1973). Ribonuclease A, ribonuclease T₁, snake-venom phosphodiesterase, pancreatic deoxyribonuclease, bacterial alkaline phosphatase and pepsin were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Polynucleotide kinase was prepared as described by Richardson (1965).

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Cellulose acetate (Oxoid) electrophoresis strips were obtained from Oxo Ltd., London E.C.4, U.K. The ion-exchange papers, Whatman Chromedia DE-81 and AE-81, and cellulose phosphate powder (Whatman P70) were obtained from Reeve Angel International Ltd., London E.C.4, U.K. The scintillator 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen was obtained from Ciba Ltd., Duxford, Cambs., U.K. γ - ^{32}P -labelled ATP was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All chemicals used were, where possible, of analytical grade and all buffer and salt solutions were sterile.

Nuclease digestion and terminal labelling of nucleotides

Histones (1 mg) were dissolved in water (0.1 ml) and the pH was adjusted to pH 7–8 (pH papers) with 1 M-Tris-HCl buffer, pH 8.5. To each sample ribonuclease T_1 (1 mg/ml; 10 μl), or ribonuclease A (2 mg/ml; 2 μl) or deoxyribonuclease I (10 mg/ml; 1 μl) was added with bacterial alkaline phosphatase (0.5 mg/ml; 5 μl). The solutions were incubated at 37°C for 1 h. Phosphatase activity was destroyed by treatment with 0.2 M-EDTA, pH 7.4 (20 μl), and 1 M-HCl (40 μl) at room temperature for 10 min (Dahlberg, 1968) and

EXPLANATION OF PLATE 1

Nucleotide 'maps' of endonuclease digests of chicken erythrocyte histone fraction V and its chromatographic components

(a), (b), (c) and (d), Ribonuclease A digests; (e), (f), (g) and (h), pancreatic deoxyribonuclease digests. Digests of the histone fraction extracted at pH 1.8 after pre-extraction at pH 2.1 are shown in (a) and (e); digests of fraction Va isolated by chromatography on Amberlite CG-50 are shown in (b) and (f); digests of fraction Vb similarly isolated are shown in (c) and (g); digests of the 'run-off' fraction (R) from the Amberlite CG-50 are shown in (d) and (h). The histone samples (1 mg) were digested with nucleases, dephosphorylated and labelled by reaction with ATP and polynucleotide kinase (see the Materials and Methods section). The nucleotide 'maps' were made by two-dimensional ionophoresis (on DE-81 paper at pH 9.7 followed by pH 2) and radioautography.

EXPLANATION OF PLATE 2

Nucleotide 'maps' of some histone preparations after digestion with ribonucleases

Ribonuclease T_1 was used to digest histones dissociated from chicken erythrocyte deoxyribonucleoprotein in: (a) 0.45 M-NaCl; (b) 0.8 M-NaCl; (c) 1.2 M-NaCl; (d) 2.0 M-NaCl. Pancreatic ribonuclease was used to digest histones extracted from calf thymus deoxyribonucleoprotein with H_2SO_4 at: (e) pH 1.8 at -1°C after pre-extraction at pH 2.65; (f) pH 1.8 at $+4^\circ\text{C}$ after pre-extraction at pH 2.65; (g) pH 0.6 at -1°C after pre-extraction at pH 1.2; (h) pH 0.6 at $+4^\circ\text{C}$ after pre-extraction at pH 1.2. The samples were digested, labelled and fractionated as described in the legend to Plate 1 and in the Materials and Methods section.

EXPLANATION OF PLATE 3

Nucleotide 'maps' of pancreatic deoxyribonuclease digests of some histone preparations

(a) Saline-washed nuclei from turkey erythrocytes extracted with H_2SO_4 at pH 2.7; (b) histones extracted from turkey erythrocytes with H_2SO_4 at pH 1.9 after pre-extraction at pH 2.1; (c) histones extracted from frog erythrocytes with H_2SO_4 at pH 0.6 after pre-extraction at pH 1.4; (d) histones extracted from rabbit thymus deoxyribonucleoprotein with 0.1 M- H_2SO_4 ; (e) histones extracted from rabbit thymus deoxyribonucleoprotein with 0.25 M- H_2SO_4 ; (f) histones dissociated from yeast deoxyribonucleoprotein in 2.0 M-NaCl. The samples, which had all been prepared at 4°C , were digested, labelled and fractionated as described in the legend to Plate 1 and in the Materials and Methods section.

EXPLANATION OF PLATE 4

Nucleotide 'maps' of turkey erythrocyte histones that had been terminally labelled

(a) Before digestion with pancreatic ribonuclease; (b) after digestion with pancreatic ribonuclease; (c) before digestion with pancreatic deoxyribonuclease; (d) after digestion with pancreatic deoxyribonuclease. The histones were extracted with H_2SO_4 at pH 1.0 after pre-extraction at pH 1.8. The samples were labelled as described in the Materials and Methods section and fractionated as described in the legend to Plate 1.

the solutions were finally neutralized with 1M-NaOH. Then 1M-MgCl₂ (10 μ l), 0.1M-2-mercapto-ethanol (20 μ l), γ -³²P-labelled ATP (1 μ mol/ml; 5 μ l) and polynucleotide kinase (phosphocellulose fraction; 15 μ l) were added to the neutralized digests and the solutions were incubated at 37°C for 3h and then either concentrated or dried *in vacuo*.

Ionophoresis

The basic procedures and the general conditions of ionophoresis on ion-exchange papers were those of Sanger *et al.* (1965) and Murray (1970). Preliminary analyses for nucleotides in the kinase reactions were done on Whatman AE-81 paper at pH3.5. Two-dimensional separations of nucleotides were done either on cellulose acetate strips at pH3.5 or on Whatman DE-81 paper at pH9.5 in the first dimension and on Whatman DE-81 paper at pH2 in the second dimension. Labelled nucleotides were detected radioautographically on Kodak Blue Brand medical X-ray film. In one series of experiments the nucleotides were eluted from the ion-exchange papers with 30% (v/v) triethylamine carbonate and subsequently analysed by complete and partial digestion with snake-venom phosphodiesterase (Murray, 1970).

Sucrose gradient centrifugation

Sucrose gradients were prepared with the apparatus designed by Britten & Roberts (1960), by supplying 1.2ml of 20% (w/v) sucrose in 0.1M-NaCl with 1.2ml of 5% (w/v) sucrose in 0.1M-NaCl. Nucleotides associated with histones (terminally labelled but not digested with nucleases) were layered on top of these gradients; in one tube ³H-labelled bacteriophage λ DNA was layered on top of the gradient. Centrifugation was at 190000g_{av.} for 4h at 4°C (MSE SS65 centrifuge, 3 $\times$ 3SW rotor, 50000rev./min). Two-drop fractions were collected on discs of Whatman GF/C glass-fibre paper (2.5cm diam.) and counted for radioactivity [0.4% (w/v) 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen in toluene (2ml)] in a liquid-scintillation spectrometer.

Phosphocellulose adsorption

Histones were dissolved in water (5mg/ml) and the solution was clarified by centrifugation. Phosphocellulose powder (Whatman P70) was then added until the E_{275} remained constant (at less than 20% of its original value) on further addition of phosphocellulose. The resulting suspension was centrifuged and the nucleotides remaining in the supernatant were labelled with polynucleotide kinase and examined by two-dimensional ionophoresis.

Results and Discussion

Our nucleotide analyses were based upon the transfer of the γ -phosphorus of ATP to the 5'-hydroxyl

groups of ribo- and deoxyribo-nucleotides by polynucleotide kinase (Richardson, 1965; Novogrodsky & Hurwitz, 1965, 1966). The sensitivity of this reaction is governed by the specific radioactivity of the [γ -³²P]-ATP; this was about 10mCi/ μ mol. The reaction was used in two ways, first to label nuclease digests (after dephosphorylation) of histone samples, and secondly to label the nucleotides in histone samples without prior nuclease digestion. The first type of experiment was initially done with or without prior digestion of the histones with pepsin, but as the results were independent of pepsin treatment this was discontinued in later experiments. After phosphorylation of the nuclease digests, the reaction products were screened by ionophoresis on Whatman AE-81 paper at pH3.5 and then examined at higher resolution by two-dimensional ionophoresis on Whatman DE-81 paper.

Plate 1 shows the results of experiments with terminally labelled nuclease digests of chicken erythrocyte histone fraction V (prepared by acid extraction at pH1.8 after extraction at pH2.1) and its chromatographic components Va and Vb. A difference in nucleotide content clearly is not responsible for the chromatographic separation of the two components and, indeed, ion-exchange chromatography has effectively separated the nucleic acid from the histones. In addition, if histone preparations were treated with phosphocellulose the material that could be labelled with polynucleotide kinase remained in the supernatant solution whereas the histones were adsorbed on the ion-exchange resin.

Several other histone samples, made by different procedures, were then examined in a similar way and complex nucleotide 'fingerprints' were obtained with all but the chromatographic fractions. The samples examined are listed in Table 1 and examples of the 'fingerprints' of ribonuclease and deoxyribonuclease digests are shown in Plates 2 and 3. Whereas chromatographic fractions were essentially free from nucleotides, the 'run-off' fraction (fraction R), obtained after chromatography of histone fraction V (Figs. 1 and 2 in Greenaway & Murray, 1971), contained nucleotides and its 'fingerprint' showed some similarities to that of the parent histone preparation (Plate 1). Nucleotide 'fingerprints' were also made from nuclease digests of the material present in the dilute salt washings of turkey erythrocytes after lysis with saponin but before extraction of histones. These 'fingerprints' and those of histones prepared from saline-washed nucleoprotein either by dissociation at various NaCl concentrations or by extraction with acid at a series of pH values showed the same general features. The close similarity of several of the 'fingerprints' (some of which are shown in Plates 2 and 3) was confirmed by elution and partial digestion with snake-venom phosphodiesterase of corresponding nucleotides. These results show that there is little

Table 1. *Histone fractions analysed for the presence of nucleotides*

Source	Histone fraction*	Preparative procedure	Nucleotides detected after digestion			
			Pancreatic ribonuclease	Ribonuclease T ₁	Pancreatic deoxyribonuclease	
Chicken erythrocytes	I	Dissociation of deoxyribonucleoprotein in 0.45M-NaCl	+	+		
	I, V	Dissociation of deoxyribonucleoprotein in 0.8M-NaCl	+	+		+
	I, V, IIb	Dissociation of deoxyribonucleoprotein in 1.2M-NaCl	+	+		+
	I, V, IIb, III, IV	Dissociation of deoxyribonucleoprotein in 2.0M-NaCl	+	+		+
	I	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH2.1 after extraction at pH2.7	+	+		+
	V	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH1.8 after extraction at pH2.1	+	+		+
	IIb	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH1.4 after extraction at pH1.8	+			+
	III, IV	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH1.0 after extraction at pH1.4	+	+		+
	'Run-off' fraction (R)	Chromatography on Amberlite CG-50	—	—		—
	V	Chromatography on Amberlite CG-50	—			—
Turkey erythrocytes	Va	Chromatography on Amberlite CG-50	—			—
	Vb	Chromatography on Amberlite CG-50	—			—
	—	Third saline wash of nuclei	+	+		+
	—	Last saline wash of nuclei	+	+		+
	—	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH2.7	+	+		+
	I	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH2.1 after extraction at pH2.7	+	+		+
	V	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH1.8 after extraction at pH2.1	+	+		+
	IIb, III, IV	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH1.0 after extraction at pH1.8	+	+		+
	V	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH1.9 after extraction at pH2.1				+
	III, IV	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH0.6 after extraction at pH1.4		+		+
Frog erythrocytes	—	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH2.65 (at 4°C)	+			+
	I	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH1.8 after extraction at pH2.65 (at 4°C)	+			+
	III, IV	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH0.55 after extraction at pH1.2 (at 4°C)	+			+
	—	Residue after extraction at pH0.55 at 4°C	+			+
Calf thymus glands	I	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH1.8 after extraction at pH2.65 (at -1°C)	+	+		+

	+	+		+	+	+
	+	+	+	+	+	+
	+	+	+	+	+	+
IIb	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH1.2 after extraction at pH1.8 (at -1°C)					
III, IV	Extraction of deoxyribonucleoprotein with H ₂ SO ₄ at pH0.6 after extraction at pH1.2 (at -1°C)					
IIb	Chromatography on Amberlite CG-50					
III, IV	Chromatography on Amberlite CG-50					
I, IIb	Extraction of deoxyribonucleoprotein with 0.1M-H ₂ SO ₄					
I, IIb, III, IV	Extraction of deoxyribonucleoprotein with 0.25M-H ₂ SO ₄					
	Extraction of deoxyribonucleoprotein with 2.0M-NaCl					
Rabbit thymus glands						
Yeast						

* Nomenclature of Rasmussen *et al.* (1962) and Murray *et al.* (1968).

or no specificity in the nucleic acids associated with the different histone fractions, or with other proteins that are present in the saline extracts.

Histone preparations were also digested with either pancreatic ribonuclease or deoxyribonuclease after the labelling reaction and fractionated by two-dimensional ionophoresis. Complex 'fingerprints' were obtained (Plate 4), indicating terminal heterogeneity in both the ribo- and the deoxyribo-nucleotides that were associated with the histones. Treatment of histone samples with phosphatase before labelling with polynucleotide kinase made little qualitative difference to the complexity of these 'fingerprints'. The distribution of label in the 5'-mononucleotides produced by complete snake-venom digestion of a terminally labelled histone sample is shown in Table 2. An estimate of the nucleotide content of this sample indicated that there was approximately 55 μ mol of terminally labelled mononucleotide/mol of histone. Accurate quantitation of the amount of nucleotide material in histone preparations can be made only if the chain length of the nucleotide is known, but clearly it will be very low. The observed amount of nucleic acid in the histone samples depended upon the method of preparation. Nuclease digests of histone samples prepared by extraction of nucleoprotein with acid contained smaller amounts of nucleotides than corresponding histone fractions prepared by dissociation of nucleoprotein with NaCl. Further, the nucleotide content of histone fractions extracted from nucleoprotein with acid at -1°C (Murray, 1969) was lower than that of corresponding extracts made at +4°C (Plates. 2 and 3). These results are explained by the much greater solubility of polynucleotides in NaCl solutions and by the temperature-dependent degradation and solubility of macromolecular nucleic acids in acid solutions.

Sedimentation of terminally labelled histone samples on sucrose gradients showed that the labelled nucleotides had a very low sedimentation coefficient (Fig. 1). From the known sedimentation coefficient of bacteriophage λ DNA, which was used as a marker in a gradient centrifuged in the same rotor, the sedimentation coefficient of the nucleotides associated with histone fractions was estimated to be less than

Table 2. 5'-Terminal mononucleotides of the polynucleotides associated with rabbit thymus histones extracted with 0.1M-sulphuric acid

	CMP	AMP	GMP	UMP
Net c.p.m.	131	1137	577	127
Equivalent μ mol/mol of histone	3.6	32	16	3.6
Approx. proportion	1	9	4	1

Fig. 1. Sucrose gradient centrifugation of the terminally labelled polynucleotides associated with histone fractions

Histone samples (1 mg) were terminally labelled by reaction with polynucleotide kinase as described in the Materials and Methods section. The labelled material was layered on a 5–20% sucrose gradient and centrifuged at $190000g_{av}$ for 4 h at 5°C. Fractions were collected and the radioactivity measured by counting in a liquid-scintillation spectrometer. The direction of sedimentation was from right to left. Δ , Rabbit thymus histones were extracted with 0.1 M- H_2SO_4 . $\bullet$, Chicken erythrocyte histones were extracted with H_2SO_4 at pH 1.0 after pre-extraction at pH 1.8.

5S. When the 3H -labelled bacteriophage DNA was included as an internal reference in the reaction mixture the ^{32}P -labelled nucleotides became associated, presumably by their histones, with the marker DNA.

The complexity of the nucleotide 'fingerprints' obtained from all the histone preparations examined, the 5'-terminal sequences of the undigested preparations and the sedimentation-velocity experiments were consistent with the nucleotides being small and heterogeneous. There was no difference in terminal labelling before or after phosphatase treatment; the nucleotides are therefore terminated with 5'-hydroxyl groups. Terminally labelled histone samples that had not been treated with phosphatase were readily degraded by snake-venom phosphodiesterase, which suggests that they may also contain 3'-hydroxyl groups. This would imply that the terminal nucleotides cannot be covalently bonded to the proteins. Since nucleotides were found in histone fractions prepared by acid extraction, the complex cannot involve acid-labile covalent bonds. Indeed, the removal of the nucleotides from histones by ion-exchange resins shows that the association is ionic.

The general conclusion is that all histone fractions prepared by acid extraction and by salt dissociation are contaminated to varying degrees by heterogeneous polynucleotides. This conclusion and those of Heyden

& Zachau (1971), Artman & Roth (1971), Hill *et al.* (1971) and Szeszák & Pihl (1972) must challenge the significance of the so-called 'unique' histone-bound RNA species and their relevance to specific repression mechanisms. The results, together with the analyses of extracts from ^{32}P -labelled cell nuclei of the Chinese hamster ovary, published by Shepherd *et al.* (1970), are also relevant to metabolic studies of histone phosphorylation. Conclusions from such studies may be invalid if the removal of contaminating nucleotides has not been established, as described by Balhorn *et al.* (1971), for example, and the phosphorylated serine or threonine sequences of the histones analysed.

P. J. G. acknowledges the receipt of a Medical Research Council studentship. The work was supported in part by a grant from the Science Research Council.

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