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CHANGES IN CHROMATIN OF *DAUCUS CAROTA* CELLS DURING EMBRYOGENESIS

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Key Word Index—*Daucus carota*; Umbelliferae; carrot; embryogenesis; non-histone proteins; template activity.

Abstract—Cells of carrot (*Daucus carota* var. Rote Riesen) were cultured on media inductive and non-inductive for embryogenesis and analyzed for differences in their chromosomal proteins and chromatin template activity. Non-histone proteins were prepared from dehistonized chromatin and their properties were investigated. Non-histone proteins proved to be acidic and associated easily with calf thymus histone. Non-histone proteins were able to counteract the inhibitory effect of histone on DNA-directed RNA synthesis *in vitro*. Almost the same rate of restoration occurred regardless of the interaction between DNA and protein, when sufficient amounts of non-histone proteins were added. However, once the histone-DNA complex was established, the restoration by non-histone proteins at comparably lower concentration was poor. Another acidic protein, bovine serum albumin, had no effect on histone inhibited RNA synthesis. Also non-histone proteins enhanced the chromatin directed RNA synthesis more than 100%. The template activity of chromatin changed after the inductive treatment of embryo formation and induced cells showed higher template activity than non-induced controls after embryo cells were formed. Histone components were the same in inductive and non-inductive cells. On the other hand, there was a correlation between template activity and the stimulation by non-histone proteins of histone-inhibited RNA synthesis.

INTRODUCTION

Recently, there has been increasing speculation concerning the role of chromosomal non-histone proteins in the regulation of transcription. It has been shown, for instance, that in *in vitro* systems for RNA synthesis, with chromatin as the template, only a part of the DNA present was transcribed [1] and this was obviously the consequence of the masking of DNA by histone. There are only a few different types of histones and the nature of histone inhibition of DNA-directed RNA synthesis is apparently non-specific [2]. Thus the genetic restriction of chromatin as well as the rate of gene transcription is most probably controlled by non-histone proteins which vary in amount and composition within tissue of the same organism during differentiation [3–5]. Also relevant are the higher rates of amino acid incorporation in non-histone proteins compared with those of the histone fraction [6], data on the interaction of progesterone with specific non-histone protein [7] and the increase of chromatin-directed RNA synthesis in the presence of non-histone proteins [8–12]. Furthermore species-specific bindings of non-histone proteins to DNA [13, 14] and the production of specific RNA by organ specific non-histone proteins have been demonstrated [15].

All these data suggesting a role for non-histone protein in gene regulations are derived from experiments with animal tissues. Comparable investigations on the non-histone proteins of plant cells are few [16]. There have been several reports on non-histone proteins related to cell development [4, 5, 16–18]. The present work developed from the discovery that the omission of the auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) from a synthetic medium induces embryo formation in carrot cells [19]. The object was to find out whether the properties of chromatin, especially non-histone proteins, will change during the early stages of embryogenesis.

RESULTS AND DISCUSSION

Chemical composition of chromatin and non-histone protein

The preparation of chromatin from tissue culture is generally more difficult than from the intact

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Table 1. Chemical composition of chromatin

Sample source	No. of preparations	DNA	RNA	Histone	Non-histone proteins
Ms	6	1	0·274 ± 0·082	1·02 ± 0·23	2·17 ± 0·42
Ms-2,4-D	6	1	0·274 ± 0·075	1·11 ± 0·26	2·38 ± 0·48

Values are expressed as means ± s.d.

plant and this is true of carrot cells. The isolated chromatin was analyzed for its DNA and RNA composition. Histone was extracted with 0·25 N HCl in ice for 30 min with occasional stirring and the rest was solubilized in 0·01 N NaOH to extract non-histone proteins [20]. Using a value of 1·0 for DNA content of chromatin, the average protein content from six samples was 3·19 in non-induced (Ms) and 3·49 in induced (Ms-2,4-D) as shown in Table 1. These values are similar to those obtained from chick brain chromatin [21].

In the non-histone protein aspartic acid was the major amino acid, followed by glycine, glutamic acid and serine (Table 2). The content of basic amino acid (arginine, lysine and histidine) was small in comparison with acidic amino acids. The ratio of acidic to basic amino acid was 1·83. This shows that carrot non-histone proteins are acidic and resemble those of animal tissues. Reported values range from 1·2 to 2·7 in rat liver [22], 1·51 in sea urchin embryo [20], 1·55 in HeLa S_3 cells [6], 1·9 in chick brain [21] and 1·4 in pig cerebellar and pituitary cells [23].

Table 2. Amino acid composition of non-histone proteins

Amino acid	Mol (%)
Aspartic acid	14·4
Threonine	4·1
Serine	10·0
Glutamic acid	10·3
Proline	5·7
Glycine	11·8
Alanine	8·2
Valine	3·7
Isoleucine	3·1
Leucine	9·4
Tyrosine	2·1
Phenylalanine	3·5
Lysine	7·6
Histidine	1·6
Arginine	4·3
Acidic/basic*	1·83

* Acidic—aspartic acid, glutamic acid; Basic histidine, arginine, lysine.

Complex formation between non-histone proteins and histone

Non-histone proteins and histone prepared from animal tissue are known to combine easily. When a solution of carrot non-histone proteins and calf thymus histone were mixed in 0·01 M Tris–HCl buffer (pH 8·0), precipitation occurred instantly. Turbidimetric determinations showed that precipitation increased with an increasing ratio of non-histone protein to histone, reaching a maximum at a ratio of 1:5 and decreasing at higher values. Wang and Johns [24] observed the combination between histone and acidic proteins from rat liver nuclei. The pattern of combination in their group pH 5 and pH 6 are somewhat similar to that observed in this work.

Complex formation between non-histone proteins and histone depends upon the salt concentration in the medium. Complex formation increased up to 0·1 M NaCl, then decreased rapidly with higher concentration of NaCl. Almost no complex formation occurred at salt concentrations higher than 0·5 M.

Restoration of histone-inhibited RNA synthesis by non-histone proteins

DNA-dependent RNA synthesis *in vitro* can be inhibited by the addition of histone to the cell free system containing RNA polymerase and DNA [25]. Histone inhibition can be reversed by the addition of non-histone proteins prepared from animal tissue [8–10, 26]. We examined the effect of non-histone proteins from carrot on the calf thymus DNA directed RNA synthesis in the presence of calf thymus histone. In the system containing 5 μg DNA, we found a distinct decrease in the rate of transcription upon the addition of 21·6 μg histone. The addition of histone resulted in a 92% inhibition of RNA synthesis. However, the inhibition of RNA synthesis was progressively overcome by the addition of non-histone proteins. In the presence of increasing amounts of these pro-

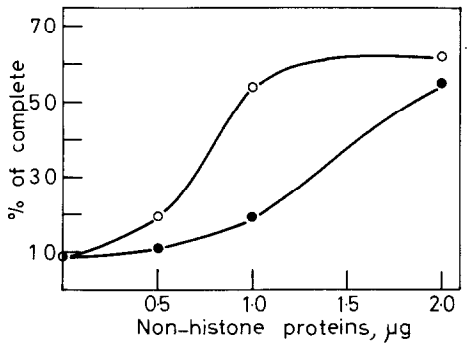

Fig. 1. Effect of non-histone proteins on histone inhibited RNA synthesis.
Histone (10 μg/tube), DNA (2·5 μg/tube). The measurements are expressed as % of the activity of the complete system (43 pmoles of [^{14}C]-AMP incorporated) in the absence of histone. ○—non-histone proteins + histone, DNA last: ●—DNA + histone, non-histone proteins last.

teins, this restoration first increased, reached a maximum and then decreased. Maximum restoration, 73% of the system without histone, was observed when 6 μg of non-histone proteins were added. This activity of non-histone proteins is very high compared with that of the similar proteins from animal tissues. In the case of rat liver, non-histone protein in amounts equal to that of the histone were required to restore the inhibition of RNA synthesis [9]. Also, non-histone proteins from rat liver strongly prevent the histone inhibition of RNA synthesis only if interacted directly with histone prior to its association with DNA. In our experiments, almost the same rate of restoration occurred regardless of how the various macromolecules were mixed together, when sufficient amounts of non-histone proteins were used. However, once the histone-DNA complex was established, the restoration by non-histone proteins at comparably lower level was poor (Fig. 1). Thus, non-histone protein may not only prevent direct DNA-histone association but also aid in the dissociation of DNA-histone complexes.

Table 3. Effect of bovine serum albumin and non-histone proteins on histone inhibited RNA synthesis

Additive (μg) to complete		RNA synthesis
Histone	Bovine serum albumin	% of complete
0	1	101·7
0	10	124·0
10	0	10·8
10	1	12·2
10	2	12·4
10	10	14·3
10	20	12·8
Histone	Non-histone proteins	
10	0·5	46·2
10	1·0	90·2

The complete system contained 2·5 μg of calf thymus DNA and other essential reaction components as shown in Experimental. Reaction was progressed with constant amount of *E. coli* RNA polymerase and 35 pmoles of [^{14}C]-AMP was incorporated in the complete system.

When histone was mixed with other acidic proteins such as bovine serum albumin under similar conditions, no effect was observed (Table 3). Even adding 20 times more bovine serum albumin than non-histone proteins had no effect. Thus the restoration of DNA dependent RNA synthesis inhibited by histone is more than the result of a simple chemical interaction between acidic and basic protein.

Activation of chromatin directed RNA synthesis by non-histone proteins

In the following experiment DNA was replaced by chromatin as template. The results in Fig. 2 indicate that transcription from chromatin as tem-

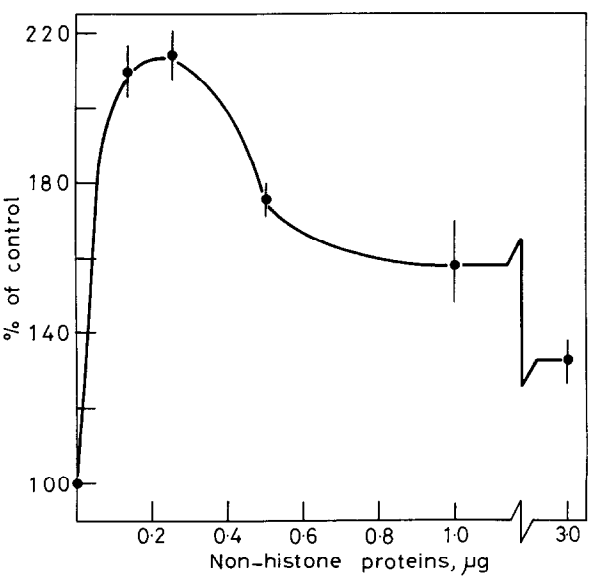

Fig. 2. Activation of chromatin directed RNA synthesis by non-histone proteins.
Chromatin containing 1·2 μg DNA was mixed with various amounts of non-histone proteins. Reaction tube contained a constant amount of *E. coli* RNA polymerase and the other requisite constituents for RNA synthesis as shown in Experimental. The measurements are expressed as % of the activity (10 pmoles of [^{14}C]-AMP incorporated) of the control system in the absence of non-histone proteins. Each point represents the average of triplicate assays and bars represent the range of values.

plate increased after the addition of non-histone proteins. When a chromatin equivalent to 1·2 μg DNA was used, RNA synthesis first increased, reached a maximum and then decreased. This increase of RNA synthesis probably depends on complex formation between the added non-histone proteins and histone in the chromatin, and this association releases DNA available as template. The addition of 0·125 μg non-histone proteins to the chromatin containing 1·2 μg DNA increased the RNA synthesis by about 110%. A similar promotion was observed in an experiment with material from animal tissues [10], but again carrot non-histone proteins had a much higher effect than those from animal tissues. In the case of spleen chromatin, the template activity was increased up to 30% by the addition of non-histone proteins in amounts equal to the DNA in chromatin [10]. The promotive effect of non-histone proteins on chromatin directed RNA synthesis was only observed when freshly prepared chromatin was used. It did not occur with chromatin kept at 0° or −20° for more than 1 day. The chromatin used as template contains its own non-histone proteins but the sharp activation of template activity was observed by the external addition of small amounts of non-histone proteins. This probably indicates that non-histone protein fractions with counteracting activity to histone inhibited RNA synthesis were concentrated during their preparation.

Table 4. Characterization of the RNA synthesis directed by chromatin

Incubation mixture	RNA synthesis (cpm)
Complete*	412
Polymerase omitted	24
UTP and CTP omitted	91
Chromatin omitted	47
Mg^{2+} and Mn^{2+} omitted	15
Actinomycin D (20 μg) added	37
Non-histone proteins†	25

* Chromatin containing 1·8 μg DNA was used as template.
† Chromatin was replaced with 15 μg of non-histone proteins.

Changes of chromatin template activity during embryo induction

Chromatin isolated from carrot cells can prime RNA synthesis in the presence of exogenous RNA polymerase under appropriate conditions. Template activity of chromatin was limited by the amount of exogenous *E. coli* RNA polymerase. Furthermore, the template activity of chromatin depended on the presence of ribonucleoside triphosphate, Mg^{2+} and Mn^{2+}. The activity was inhibited by actinomycin D. When the chromatin was replaced by 15 μg of non-histone protein, no RNA synthesis was detected (Table 4). This indicates that non-histone proteins have no template activity. When chromatin prepared from 14-day-old cultures was used, RNA synthesis progressed proportionally with the increasing concentration of the template (Fig. 3).

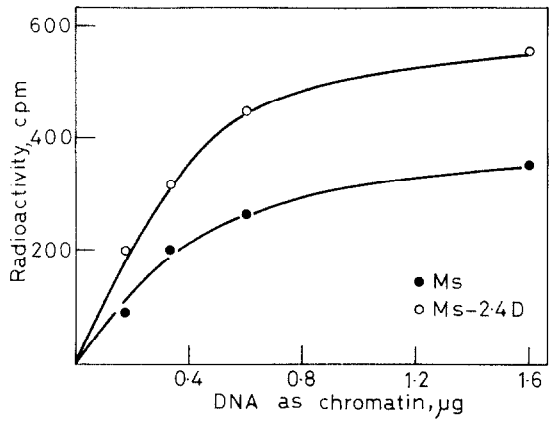

Fig. 3. Rate of RNA synthesis directed by chromatin as a function of template concentration.
Reaction mixture contained various amounts of DNA as chromatin, constant amount of *E. coli* RNA polymerase and other requisite constituents for RNA synthesis, as described in the Experimental.

Table 5. Incorporation of [^{14}C]-AMP into RNA synthesized on chromatin as template

Sample source	Age of sample: 6 days (A)	6 days (B)	6 days (C)	14 days (A)	14 days (B)	14 days (C)
Ms	0·6	283	472	0·6	270	450
	1·2	350	292	1·2	350	292
Ms-2,4-D	0·6	279	465	0·6	450	650
	1·2	336	280	1·2	530	442

Chromatin was prepared from carrot cells cultured with and without 2,4-D. Reaction was progressed with constant amount of *E. coli* RNA polymerase and other requisite constituents for RNA synthesis as shown in Experimental for 30 min at 37°. (A) chromatin (μg of DNA equivalent); (B) radioactivity incorporated into RNA (cpm); (C) radioactivity incorporated into RNA (counts/min/μg of DNA equivalent).

The template activity of chromatin prepared from 6 to 14 days after induction of embryogenesis was different in embryo-forming cultures from

embryo-free controls (Table 5). Six days after the omission of 2,4-D, there was still no difference in the template activity of chromatin but 14 days after the omission of 2,4-D, it was 45% higher in the embryo forming cells.

It has been postulated that cell and tissue differentiation are principally correlated with restriction of genetic transcription and it is generally observed in eukaryotic cells that the template activity of chromatin changes with the different stages of morphogenesis in animals [3, 11, 27] and in plants [28, 29]. In order to evaluate correlations between embryo formation and changes of template activity, it is necessary to consider the different stages of development after induction of embryogenesis.

A few days after the omission of auxin, parenchymatic cells were converted to meristematic cells which produced globular cells lacking vacuoles. One week later the cells began to form actively dividing heart shaped embryos with high metabolic activities. At the same period, only parenchymatic cells with many vacuoles were observed in the control. The high template activity after 14 days of inductive treatment was at least partly the consequence of the different physiology of embryo induced cells.

In this work, embryo formation and the changes in template activity were induced by the omission of 2,4-D; according to several recent publications, auxin can influence RNA synthesis positively *in vivo*, and *in vitro* in the presence of particular protein factors which associate with hormone [30–32]. It has been postulated that IAA, once it has formed a complex with an acceptor protein, combines with DNA at a positive site and then associates with the initiation factor and RNA polymerase [16]. Similarly another auxin, 2,4-D first releases a transcriptional factor from the plasma membrane and this is then transported into the nucleus where it regulates the activity of RNA polymerase [33]. In our preparations, the chromatin had almost no RNA polymerase activity and RNA synthesis was measured at saturating level of exogenous *E. coli* RNA polymerase. The promotive actions of auxins in RNA synthesis are based on the substantial regulation of plant RNA polymerase in all cases [16, 33]. This excludes the possibility that enzyme activities are involved in the changes of template activities observed in present work. The addition of 2,4-D (10^{-5} to 10^{-7} M) showed almost no effect on chromatin directed RNA synthesis *in vitro*. Therefore the increase of template activity after the omission of 2,4-D cannot be due simply to changes in auxin levels, but rather to the effect of embryogenesis.

Changes in activity of non-histone proteins in histone-inhibited RNA synthesis

Investigations of histone at different stages by polyacrylamide gel electrophoresis showed that the pattern of histone was not altered during embryogenesis. Then RNase activity of chromatin was checked according to the method used for barley RNase [34] and no RNase activity was found in chromatin prepared from both types of carrot cultures. These results show that neither of these factors change template activity. In contrast, changes of the electrophoretic pattern of non-histone proteins were observed (unpublished results). It is generally accepted that non-histone proteins have a gene regulative activity through their interaction with histone [8–11]. There have been several reports showing the important role of non-histone proteins in the regulation of template activity of chromatin [26, 35]. It was also observed in this experiment that carrot non-histone proteins prevent histone inhibition of RNA synthesis. Therefore, the activity of non-histone proteins prepared from induced and non-induced samples were examined and found to be almost the same after 6 days of treatment (Fig. 4). On the other hand, the non-histone proteins prepared from induced cells after 14 days of the treatment restored RNA synthesis more than those of non-induced cells. About 0·7 μg of non-histone proteins of induced cells is required for 50% restoration but 1·2 μg is needed for the same restoration from the non-induced cells. When these results are compared with the template activity observed in chromatin (Table 5), it can be seen that there is a correlation. This shows that qualitative changes of non-histone proteins together with changes in their stimulation of histone-inhibited RNA synthesis occurred during the formation of different cell types. The higher template activity might be caused by the greater activity of the non-histone proteins. Correlations between the template activity of chromatin, the capacity of non-histone proteins to restore histone-inhibited RNA synthesis

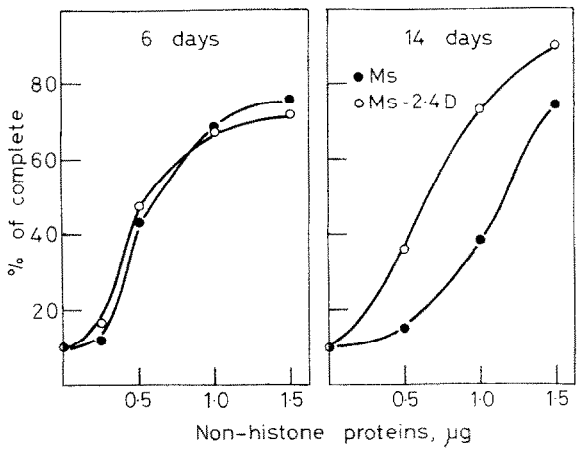

Fig. 4. Counteracting activity of non-histone proteins to histone inhibited RNA synthesis.
Calf thymus DNA (2·5 μg) was mixed with different amounts of non-histone proteins and then 10 μg of calf thymus histone was added. The reaction tubes contained a constant amount of *E. coli* RNA polymerase and other requisite constituents for RNA synthesis. The measurements are expressed as % of the activity (56 pmoles of [^{14}C]-AMP incorporated) of the complete system in the absence of histone.

and tissue specificity in the promotion of transcription have been noted in animal systems [9, 10, 26]. The amounts of non-histone proteins prepared from induced and non-induced samples differed slightly and this could be another factor in the regulation of template activity [36, 37].

EXPERIMENTAL

Cultural methods. The carrot (*Daucus carota* var. Rote Riesen) cultures used in all experiments were derived from surface sterilized main roots. Cultures were started from small explants of the upper third of the main root and were placed on Ms 0·8% (w/v) agar medium which contained the major and minor elements of the medium developed by Murashige and Skoog [38] and the organic matter according to White [39] and in addition 2% (w/v) sucrose and 2,4-D (5×10^{-8} g/ml). The transfer period was always 4 weeks. After 10–14 months the cells were transferred from agar to suspension culture medium containing the same substances but no agar and were cultured on rotatory shakers for 2 weeks. Subsequently the cells were collected and transferred to two different culture media: one was Ms (non-inductive) and the other Ms without 2,4-D "Ms-2,4-D" inductive form for embryogenesis. Only the materials from Ms were used in the work concerning general properties of non-histone proteins.

Preparation of chromatin. The cells (50–200 g fr. wt) were collected and washed with H_2O. They were homogenized in a Waring blendor for 1 min at max. speed with × 1·5 vol. of grinding medium consisting of 0·25 M sucrose, 0·05 M Tris–HCl buffer (pH 8·0), 0·001 M $MgCl_2$ and 0·01 M β-mercaptoethanol. The homogenate was then treated in a Potter homogenizer. The material was subsequently filtered through cheese cloth and Miracloth. The filtrate was centrifuged at 4000 *g* for 30 min at 4°. The ppt. was washed with grinding medium (× 1) and with 0·05 M Tris–HCl buffer (pH 8·0) containing 0·01 M β-mercaptoethanol (× 4), each time followed by centrifugation at 10000 *g* for 10 min at 4°. The final ppt. was homogenized in a Potter homogenizer and layered on a 1·7 M sucrose in 0·05 M Tris–HCl buffer (pH 8·0). Sample in a SW-25 spinco head was centrifuged at 70000 *g* for 105 min at 4°. Chromatin from carrot cells prepared by this method [34, 40] was found to be contaminated with non-chromosomal proteins and the ratio of protein to DNA often showed a value of more than 5. Therefore, instead of the centrifugation through 1·7 M sucrose, the chromatin suspension was layered on a discontinuous sucrose gradient consisting of 5 ml of 1·5 and 1·7 M, 12 ml of 2·0 M and 3 ml of 2·5 M sucrose. Tubes were centrifuged as before. After centrifugation, two layers of 2·0 and 2·5 M sucrose fractions were collected, leaving small amounts of starch and other materials on the bottom of the tube, and made up to 180 ml with 0·05 M Tris-HCl buffer (pH 8·0). Then the sample was centrifuged at 30000 *g* for 20 min at 4°. The clear ppt. was collected, homogenized in a Potter homogenizer and layered on a second sucrose gradient consisting of 10 ml of 1·7 M and 15 ml of 2·0 M sucrose and centrifuged as before. Finally the chromatin ppt. was homogenized in 0·01 M Tris–HCl buffer (pH 8·0) and dialyzed against the same buffer overnight at 4°.

Preparation of histone and non-histone proteins. The dialyzed chromatin was first treated with 0·4 N HCl in ice for 10 min to remove histone and centrifuged at 30000 *g* for 15 min at 4°. The ppt. was again treated with 0·25 N HCl for 15 min and centrifuged as before. Both HCl extracts were combined and dialyzed against H_2O. The dialyzed sample including histone was lyophilized. The acid insoluble materials were dissolved in 7 ml of 0·05 M Tris–HCl buffer (pH 8·0) containing 1·0% (w/v) SDS, carefully adjusted to pH 8·0 with NaOH and stirred overnight at 28° to solubilize non-histone proteins and DNA [41]. The solubilized materials were centrifuged at 110000 *g* for 25 hr at 18°. After centrifugation, the upper 75% of the supernatant was pipetted out and dialyzed for 5 hr against 0·01 M Tris–HCl buffer (pH 8·0) containing 0·1% (w/v) SDS at 4°. To the dialyzed sample 2–3 drops of satd KCl were added. The resulting dense ppt. of potassium dodecyl sulfate in ice was sedimented for 20 min at 4° at 30000 *g*. The clear supernatant was decanted and $(NH_4)_2SO_4$ was added to 60% saturation. The protein was allowed to flocculate for 30 min and was collected by centrifugation. The protein was dissolved in 0·01 M Tris–HCl buffer (pH 8·0) and dialyzed against the same buffer. With this method in four different experiments, average values of non-histone proteins prepared from 100 g of cells were 102 μg in Ms and 117 μg in Ms-2,4-D sample respectively. The recovery of non-histone proteins was about 30% of the original non-histone proteins in chromatin.

Electrophoresis of histone. The polyacrylamide gel electrophoresis was done according to the method of Bonner *et al.* [40]. Electrophoresis was from anode to cathode with load of about 100 μg of histone in a solution containing 10 M urea. Samples were separated in 15% (w/v) polyacrylamide gel containing 6 M urea at pH 4·3. Gels were stained with amido-black after electrophoresis and protein bands were recorded at 570 nm on a densitometer.

RNA polymerase assay. The standard assay tube (1·0 ml) contained Tris-HCl buffer (pH 8·0) 40 μmol, $MnCl_2$ 1 μmol, $MgCl_2$ 4 μmol, GTP, CTP and UTP (Boehringer) 0·2 μmol each, ATP-8-^{14}C (Amersham, sp. act. 53 μCi/mmol) 0·2 μCi, β-mercaptoethanol 12 μmol, 1–2 units of *E. coli* RNA polymerase (Boehringer) and appropriate amount of highly polymerized calf thymus DNA or sheared chromatin as template. Chromatin was sheared in a VirTis 45 homogenizer at medium speed for 60 sec. About 70% of DNA was solubilized by this treatment.

To this system calf thymus histone was added in the tests of the protective effect of non-histone proteins on histone inhibition. The reaction mixture was incubated for 30 min at 37°; then 1·0 ml of 25% (w/v) TCA and 50 μg of bovine serum albumin were added. After 30 min incubation in ice a sample was collected on a membrane filter (0·45 μm) and washed ×6 with 5 ml of cold 5% TCA. The washed membrane was dried at 70° for 30 min, introduced into 10 ml of toluene system scintillator and counted with Nuclear Chicago scintillation counter model 6860.

General methods. Protein was assayed by the method of Lowry *et al.* [42]. RNA was determined in the 0·3 N KOH hydrolyzable (37°, 5 hr) material by the orcinol reaction [43], and DNA determined in the hot 5% PCA hydrolyzable (90°, 15 min) material by the diphenylamine method [44]. Non-histone proteins for amino acid analysis were dissolved in 6 N HCl, placed in vial which was flushed with N_2 and incubated at 110° for 24 hr. The hydrolysates were washed ×5 with H_2O which was evaporated, dissolved in 0·2 M citrate buffer (pH 2·2) and chromatographed on a Labotoron Chromatocord.

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