Studies on the Infection and the Multiplication of Plant Viruses

IV. Suppression of cellular RNA synthesis by the cucumber mosaic virus-infection*

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加藤 盛**・三沢正生** : 植物ウイルスの感染と増殖に関する研究

IV. CMV 感染による細胞 RNA 合成の抑制*

Abstract

1) The cellular RNA content was altered by CMV infection. The amount of RNA in the infected cells decreased during the first 24 hr after infection, and thereafter increased gradually. It is conceivable that the reduction in RNA content in the early stage of infection resulted from the suppression of cellular RNA synthesis, and that the increase in RNA in the later stage was due to the increase of viral RNA in the cells.

2) Base composition and buoyant density of DNA were not significantly altered by infection. The suppression of cellular RNA synthesis by CMV seems not to be caused by a compositional change of DNA.

3) The rate of histone synthesis changed concurrently with the changes in the rate of DNA synthesis. The change in histone synthesis was not only quantitative but also qualitative; the fractionation of histone revealed a preferential increase in arginine-rich histone fraction.

4) RNA synthesis in isolated calf thymus nuclei was suppressed by the addition of tobacco histone. This inhibitory effect was greater in histone from infected cells than in that from normal cells.

5) These results suggest that the suppression of cellular RNA synthesis by infection is due to the compositional change of histone.

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Introduction

It has been shown that the synthesis of normal cell RNA and protein is inhibited in poliovirus infection of HeLa cells^{8,9}, mengovirus infection of mouse cells⁴ and others^{3,6,7,21}. There are many reports that have been concerned with the mechanism for the suppression of cellular RNA synthesis evoked by viral infection. Baltimore and Franklin⁴ and Holland⁹ found that the aggregate enzyme (DNA-protein complex containing RNA polymerase)²² activity is reduced in the virus infected cell. Holland⁹ reported that failure of polymerase synthesis alone cannot be the sole explanation for the repression of normal RNA synthesis in poliovirus

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infected cells. Furthermore, he believes that depression of cell RNA synthesis cannot be attributed to DNA breakdown, nor to a film masking the priming ability of DNA, and that the production of loosely bound material capable of masking cannot be ruled out. The precise mechanism for the suppression of the normal cellular RNA and protein synthesis in the cells infected with viruses is not fully understood. Huang and Bonner¹⁰⁾, working with pea seedling, have postulated that the histone with which DNA is combined in the genetic material of higher organisms has as one of its roles the regulation of DNA-dependent RNA synthesis. In subsequent papers, Allfrey *et al.*²⁾, working with calf thymus histone, reported similar results.

Previous work¹⁷⁾ in our laboratory demonstrated a temporary increase of the histone in tobacco leaf cells soon after infection.

The purpose of this investigation was to determine if the histone plays a role in the regulation of cellular RNA synthesis in virus-infected tobacco tissue.

Materials and methods

Test plant

Tobacco plants (*Nicotiana tabacum* var. Bright Yellow) which were grown in a growth cabinet (day at 28°C and night at 25°C at about 80 % humidity; intensity of illumination, 12,000 lux; day length, 12 hr) were used at the 15th leaf stage.

Virus

The virus used in the experiment was the ordinary strain of cucumber mosaic virus (CMV). Inoculum was obtained by grinding and squeezing the inoculated tobacco leaves with a 0.1 M phosphate buffer at pH 7.0. Inoculation by CMV was applied on the upper surface of the expanded leaves of tobacco plants by the carborundum method. For the control, identically grown plants were rubbed with the healthy leaf extract in the same manner. Control and inoculated plants were held in the growth cabinet as cited above.

Extraction and determination of whole RNA in the cell

The RNA of tobacco leaves was extracted in cold PCA according to the method of Ogur and Rosen¹⁹⁾ and the amount was determined by the orcinol reaction¹²⁾.

Preparation of DNA

Tobacco leaves were ground in a glass homogenizer. DNA was extracted by a modification of the method of Marmur¹⁴⁾; in order to remove proteins, CHCl_3 -octanol and phenol were used before the addition of 1 M NaClO_3 , and further deproteinization was made with CHCl_3 -octanol (24:1 v/v). The purine and pyrimidine composition of DNA was determined by the method of Marshak and Vogel¹⁵⁾.

CsCl density gradient centrifugation of DNA

The DNA was centrifuged in a CsCl density gradient for 48 hr at 36,000 rpm in the RPS 40 rotor of a Hitachi 55 P centrifuge. After centrifugation, the bottom of the tubes was punctured to obtain contents. The optical density of the material at 260 m μ was measured by a spectrophotometer.

Estimation of histone-decomplexed DNA (free DNA)

The free DNA was estimated by subtracting the DNA in the nucleoprotein from the total chromatin DNA values. The nucleoprotein was prepared from the chromatin fraction following the method of Zubay and Doty²⁸⁾.

Measurement of histone in the nucleus

Samples were excised from inoculated leaves successively after the inoculation. The

Fig. 1. Isolation of chromatin.

samples were fixed for 24 hr in 10 % neutral formalin, washed overnight in running tap water, dehydrated and embedded in paraffin and cut into sections of 10μ width. These sections were stained with Fast green FCF by the method of Alfert and Geschwind¹⁵. The measurements were made on 400-500 nuclei of parenchyma cells after the two wavelength method of Mendelson¹⁶, using a microspectrophotometer (Olympus Co. model IV).

Preparation of histone

For the preparation of tobacco leaf chromatin the method of Huang and Bonner¹⁰ was used with a slight modification as shown in Fig. 1. The isolated chromatin was suspended in cold 0.01 M Tris-HCl buffer (pH 8.0), and stirred in ice water to which one-fourth volume of cold 1 N H_2SO_4 was added slowly. After 30 min the sediment was broken up and extracted with 0.4 N H_2SO_4 (one-half the final volume of the first extract) and the histone sulfate precipitated quantitatively at $-20^\circ C$ for 36 hr. The histone sulfate was recovered by centrifugation, washed three times with ethanol, and dried in a vacuum desiccator.

Amino acid analysis

Histone samples (3mg) were hydrolyzed in 2 ml of constant boiling 6 N HCl solution in evacuated, sealed tubes for 22 hr at $105^\circ C$. The amino acid analysis was performed using a Hitachi automatic amino acid analyzer.

Disk electrophoresis of histone

Disk electrophoresis of histone was carried out by the method of Reisfeld *et al.*²⁰ Acid-

extracted histones were dissolved in 2 parts β -alanine-acetic acid buffer (3.2 % β -alanine and 0.8 % glacial acetic acid) at pH 4.0, and one part 1.7 M sucrose. A sample was applied to a gel. Acrylamide gel was made from 0.5 % of tetraethylenediamine, 15 % acrylamide, 0.1 % methylenbisacrylamide with 0.14 % ammonium persulfate as catalyst. Electrophoresis was carried out at a constant current of 5 mA per tube, for 1.5 hr at room temperature. Gels were stained by 7 % acetic acid solution containing 1 % amidoblack 10 B. After staining, the gels were destained by electrophoresis in 7 % acetic acid solution. The histone bands of gels were photographed. Films were printed and subjected to densitometric analysis.

Fractionation of histone

Histone was fractionated on a carboxymethylcellulose column after the method of Johns *et al.*¹¹⁾ The histone was dissolved in buffer (pH 4.2) containing 0.1 M acetic acid and 0.03 M NaOH, and then applied to a column. Elution was carried out in four steps as follows: (1) a buffer (pH 4.2) containing 0.1 M acetic acid and 0.03 M NaOH, (2) a buffer (pH 4.2) containing 0.17 M acetic acid, 0.051 M NaOH and 0.42 M NaCl, (3) 0.01 N HCl, and (4) 0.02 N HCl. Each elute of 5 ml was collected automatically at a flow rate of about 0.5 ml/min. Protein content in each elute was determined by absorption at 280 m μ or by the method of Lowry *et al.*¹³⁾

Preparation of labeled tobacco histone

Tobacco disks were cut from leaves with a cork borer and were incubated for 6 hr in Petri-dish, each of which contained distilled water containing 0.1 μ C/ml of L-¹⁴C-leucine. After the incubation, the leaf disks were washed with distilled water. Chromatin was isolated by the method of Huang and Bonner¹⁰⁾. Untreated leaves were added as a carrier. Histone was isolated from the purified chromatin as described above and then fractionated on a carboxymethylcellulose column. The histone in each fraction was precipitated by trichloroacetic acid. The precipitates were washed 4 times with 5 % trichloroacetic acid; this was followed by 3 washes with 80 % acetone and for radioactivity measurements, an aliquot of each 0.5 ml was transferred to scintillation vials. The vials were filled with 15 ml of scintillator solution containing 4 g of 2, 5-diphenyloxazol (PPO), 50 mg of 1,4-bis-2- (5-phenyl-oxazolyl)- benzene (POPOP), 120 g of naphthalene, 20 g of Cab-O-Sil (Packard) and dioxane per liter. The radioactivity was measured with a liquid scintillation spectrometer (Packard 2311).

Effect of histone on the RNA synthesis

All experimental procedures employed were similar to those described by Allfrey *et al.*²⁾, except that tobacco histone was substituted for thymus histone.

Results

Suppression of cellular RNA synthesis in CMV infected tobacco cells

In a previous paper¹⁷⁾ it was shown that the CMV-infection altered nucleolar RNA synthesis. In the present experiment, the RNA content was studied using CMV infected leaves. The results are shown in Fig. 2. The RNA content of infected cells decreased about 40 % during the first 24 hr after the inoculation and thereafter increased gradually. But, the level of RNA remained below that of the control value and began to decrease again after 96 hr. The increase in the amount of RNA after 24 hr appears to be due to the increase of viral RNA within the infected cells, but not due to the restoration of cellular RNA synthesis. Since unpublished data in our laboratory indicate that most RNA synthesis during CMV infection is in fact virus-directed, it appears likely that immediately after infection cellular RNA synthesis is inhibited irreversibly in the cell.

Table 1. Base composition of nuclear DNA from healthy and infected tobacco leaves

Isolated DNA	Molar ratios calculated to a total of 100				
	Adenine	Guanine	Cytosine	Thymine	A+T/G+C
Healthy	31.0	19.5	20.1	29.4	1.52
8 hr after inoculation	32.1	18.6	17.7	31.6	1.75
72 hr after inoculation	31.9	19.5	18.2	30.4	1.65

Table 2. Proportions of DNA present in tobacco cell nucleus as the histone-complexed and histone-decomplexed (free) forms

Time after inoculation	Fraction	Histone content ^{a)}	DNA content ^{a)}	Histone/DNA	Free DNA content ^{a)}	Histone complexed DNA/Total DNA
Control	Nucleo-protein	13.8	9.71	1.42	2.15	—
	Nucleus	14.0	11.86	1.18		
10 min	Nucleo-protein	13.3	9.23	1.44	2.46	0.78
	Nucleus	13.1	11.69	1.12		
8 hr	Nucleo-protein	17.5	12.00	1.46	2.57	0.82
	Nucleus	17.5	14.57	1.20		

^{a)} μg per 10 g fresh weight.

Fig. 2. Change of cellular RNA content in infected tissue.

Base composition of DNA in the CMV infected cells

The base composition of nuclear DNA was characterized by a high content of adenine and thymine (Table 1). As shown in Table 1, the purine and pyrimidine composition of DNA from the infected tobacco leaves did not differ from that of the healthy leaves. These results suggest that the infection does not significantly alter the purine and pyrimidine composition of DNA over the period of 72 hr after infection.

The results of the CsCl density gradient fractionation of DNA are shown in Fig. 3. The buoyant density of the nuclear DNA isolated from healthy and infected leaves ranged from 1.695 to 1.699 g/ml. The average value in 5 experiments was 1.696 g/ml, and the buoyant density between the healthy and infected leaf DNA did not differ significantly. Furthermore, the increase in DNA occurring shortly after infection was bounded with histone. Free DNA, i. e., DNA not combined with histone, did not increase for 8 hr (Table 2). From these results, it appears that the suppression of host cell RNA synthesis during early stage of the infection is not due to a change of DNA.

Changes of histone content in CMV infected cells

Experiments were conducted by utilizing a microspectrophotometric method to determine if the histone content in the nucleus is altered by the infection. The results are shown in

Fig. 3. Density gradient centrifugation in CsCl of nuclear DNA from tobacco leaves.

The DNA was extracted from healthy and infected leaves. Centrifugation was run for 48 hr at 36,000 rpm in the RPS 40 rotor of Hitachi model 55 P centrifuge at 25°C. The absorbance at 260 mμ was measured after the collection of fractions throughout the gradient. Density increase toward the left.

————: DNA isolated from healthy leaves.
: DNA, 8 hr after inoculation.

Fig. 4. Histogram showing distribution of nuclei containing various amounts of histone at each period after the inoculation.

Fig. 4. Within 2 hr following the inoculation, the histone content did not change. Namely, in this period, there appears to be no significant difference in the content of nuclear histone between the infected and normal cells. The peak of histone in both the infected and healthy cells was equally evident at 200-300 arbitrary units. After 4 hr, however, the histone content in the infected cells increased significantly and the peak shifted to 400-500 units. By the end of 24 hr, it returned again to the distribution pattern similar to that of the normal cells. The change in the histone content of the nucleus occurred concurrently with the change in the DNA content¹⁷⁾. However, the alteration of histone synthesis was not only quantitative but also qualitative; the fractionation of histone on carboxymethylcellulose column showed an increase in the arginine-rich fractions. The results of a fractionation with the whole histone preparations isolated from the healthy and infected leaves are shown in Fig. 5. The peak fX contained only about 1% of the total nitrogen taken, and this initial peak was considered to be a contaminant¹¹⁾. Step (2) then removed the lysine-rich histone as two peaks, f1A and f1B. The yield of the arginine-rich histones, f3 and f4, was about 30% of the total histone isolated from the healthy leaves taken for chromatography, and about 40% at 72 hr after infection. The ratio of the protein in the arginine-rich fraction to the protein in the lysine-rich fraction of the histone isolated from the healthy leaves was 0.6, whereas that of the infected leaves increased as the infection process proceeded; it was 0.8, 0.9 and 1.0 after 5, 20 and 72 hr respectively (Table 3). These compositional changes were also

Fig. 5. Elution profile of histone from CMC column of histones isolated from incubated leaf disk.

Elution scheme used: (1) 0.1 M sodium acetate buffer, pH 4.2; (2) sodium acetate-NaCl buffer, pH 4.2; (3) 0.01 N HCl, and (4) 0.02 N HCl.

Protein concentration of each fraction was determined by optical density at 280 mμ. (A): healthy leaves histone, (B): infected leaves histone, 8 hr after inoculation, (C): 12 hr after inoculation, (D): 72 hr after inoculation.

Fig. 6. Densitometric scanning of histone bands in polyacrylamide gels electrophoresis.

Disk electrophoresis was performed in 15% polyacrylamide gels containing 6 M urea. Gels were stained with amidoblack and destained electrophoretically. A shows the electrophoretic fractionation of healthy leaf histone. B to D, the electrophoretic fractionation of histone isolated from infected leaves 8, 12 and 72 hr after inoculation respectively. Peak at far left indicates the origin of the gel.

confirmed by disk electrophoresis on polyacrylamide gels. These results are shown in Fig. 6. Similar results were obtained from the analysis of amino acid composition of the histone isolated from CMV infected cells as shown in Table 4. Although the hydrolysis of histone resulted in a partial degradation of serine and threonine, their losses were relatively so small that no correction was made for them. The histone isolated from the healthy and infected tobacco leaves equally contained about 21-24% basic amino acid, principally lysine and arginine. Lysine to arginine ratios in the histone isolated from the infected and healthy leaves were approximately 1:5 and 1:8, respectively. The arginine content in histone was found to be markedly increased in the infected cells.

Table 3. Protein content of the fraction from histone

Time after inoculation	Fraction 1, 2	Fraction 3, 4	F 1, 2/F 3, 4
Control	233.1 μ g	139.8 μ g	0.6
5 hr	199.0	168.5	0.8
20 hr	180.0	170.0	0.9
72 hr	178.6	180.3	1.01

The protein content in each fraction is taken as a measure of the fresh weight (250 g). Protein was estimated by Folin reagent.

Table 4. Amino acid composition of histone (moles %)

Amino acid	Healthy tobacco histone	Infected tobacco histone ^{a)}
Aspartic acid	6.8	6.4
Glutamic acid	9.6	9.4
Glycine	9.0	8.4
Alanine	11.5	11.1
Valine	7.4	7.1
Leucine	6.6	6.0
Isoleucine	4.7	4.1
Phenylalanine	2.3	2.7
Tyrosine	3.5	2.9
Serine	6.1	6.2
Threonine	6.2	6.2
Proline	4.1	3.3
Methionine	1.1	0.8
Cystine	0.1	+
Arginine	6.8	9.2
Histidine	1.9	2.1
Lysine	12.3	14.1
Lysine/arginine	1.8	1.5
Basic/acidic	1.28	1.60

^{a)} Histone isolated from tobacco leaves infected with CMV, 24 hr after inoculation.

Incorporation of ¹⁴C-amino acid into the nuclear histones

The data for the incorporation of ¹⁴C-labeled amino acid into the histone fractions of the chromatin isolated from CMV-infected cell nuclei fraction are shown in Fig. 5. In the increasing stage of DNA during the first 12 hr after infection, all histone fractions were labeled. The radioactivities found in the histone of fractions 3 and 4 were much greater than those of the controls. But, in the stationary stage (after 72 hr), only fractions 3 and 4 were labeled like the histone fractions from the control cells. That the synthesis of histones in fractions 1A and 1B related directly with the DNA increase may be indicated by the above experiments. Thus, the histones of fractions 3 and 4 are actively synthesized, especially in the early stage of infection.

Effects of added histone on the RNA synthesis

A study was conducted to determine whether or not the histone inhibit RNA synthesis. Since it was difficult to isolate the nuclei in a pure from tobacco leaves, calf thymus nuclei were used in the present study. The synthesis of RNA in isolated calf thymus nuclei was followed using ¹⁴C-adenosine. The incorporation of this labeled compound into nuclear RNA was allowed to proceed for 30 min at 37°C in the incubation medium. When tobacco histones were added to the incubation medium, the synthesis of RNA was inhibited slightly (Fig. 7).

Fig. 7. The effects of some histone on RNA synthesis in isolated thymus nuclei.

The amount of histone added is plotted against the relative specific activity of the total nuclear RNA.

●—● : Healthy tobacco histone.

○—○ : Calf thymus histone.

△—△ : CMV infected tobacco histone, 12 hr after inoculation.

□—□ : CMV infected tobacco histone, 72 hr after inoculation.

However, the inhibitory effect of the histones isolated from the healthy tobacco cells differed from that of CMV infected cells. The histone isolated from the CMV-infected cells inhibited RNA synthesis much more greatly than the histone from the normal cells.

Discussion

The results obtained in the present study show that the host cell RNA content is reduced by 40 % or more during the CMV infection. The reduction in cellular RNA content may result from 3 possibilities : 1) qualitative and quantitative changes in the DNA which primes RNA synthesis ; 2) regulation of enzymes in the RNA synthesizing system, and 3) degradation of RNA by enzymes such as ribonucleases.

The results of the present study indicate that the suppression of the host cell RNA synthesis induced by the CMV-infection is neither due to quantitative change of DNA nor to qualitative alteration of DNA. These results are consistent with those reported by Holland⁹⁾ in poliovirus-infected HeLa cells.

Previous work¹⁷⁾ in our laboratory has shown that the amount of DNA in the nucleus increased temporarily after CMV-infection and also that the amount of DNA in the nucleus changed concurrently with the change of histone content. Huang and Bonner¹⁰⁾, working with histone isolated from pea seedling, indicated that the DNA fully combined with an appropriate histone was not transcribed by RNA polymerase. Since the results of the present study showed that the free DNA, not combined with histone, did not increase and that only the DNA fully combined with histone increased, it is not likely that the increase of the DNA-histone complex in the nucleus is linked to the suppression of cellular RNA synthesis.

Furthermore, as reported previously¹⁸⁾, the increase of ribonuclease activity in tobacco leaf does not appear to be a specific and necessary concomitant of the infection process. Stimulation of ribonuclease activity, regardless of its cause, did not reduce cellular RNA content. Eventually, the increase in ribonuclease activity of infected cells does not seem to explain the observed depression of RNA synthesis.

Huang and Bonner¹⁰⁾, and Allfrey *et al.*²⁾ reported that the synthesis of RNA in nucleus

was regulated by histone and that when the histone was removed, the synthesis of RNA increased. Chalkley and Maurer⁵⁾ classified histones into 2 groups on the basis of their different biochemical properties: one group, named "fractions 3 and 4", has a fast turnover without DNA replication; and the other, named "1A and 1B fractions", has no turnover except during DNA replication. The 3 and 4 fractions contain arginine-rich histone¹¹⁾. In addition, it has been shown that arginine-rich histone had a greater inhibitory effect on RNA synthesis as compared with other types of histones²⁾. The histone which was altered by the CMV-infection corresponds to a fast turnover histone, since the histone isolated from infected cell had a much greater inhibitory effect on RNA synthesis than the normal one. In virus-infected cells, the DNA-primed RNA polymerase might be prevented from contact with DNA by the modified histone that occurs during infection. It is our hypothesis that the suppression of normal cell RNA synthesis by viral infection is due to the compositional change of histone which is promptly induced by the viral infection.

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和文摘要

植物ウイルスの感染と増殖に関する研究

IV. CMV 感染による細胞 RNA 合成の抑制

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CMV 感染タバコ葉細胞における RNA 合成の抑制について検討した。

1. CMV 感染タバコ葉細胞の RNA 含量は感染直後から24時間までは急速に低下するが、それ以後は漸増する。この増加は主としてウイルス RNA の合成増加によるものと思われる。
2. 細胞核 DNA の塩基組成, buoyant density は CMV の感染によってまったく変化しない。したがって感染細胞の DNA 依存の RNA 合成の低下は, DNA の質的变化によるものではないと推定される。
3. 感染細胞でのヒストン合成は DNA 合成に伴って変化するが, ヒストンは DNA と異なり, 量的だけでなく, 質的にも変動する。すなわちヒストンの各分画中, 感染によって高アルギニンヒストンの分画がいちじるしく増加してくる。
4. タバコのヒストンは calf thymus の細胞核系での RNA 合成を阻害する。阻害効果は健全細胞より感染細胞からのヒストンで顕著である。

以上の諸結果から, CMV 感染細胞でみられる細胞の RNA 合成の抑制は, 感染によるヒストンの質的な変動に起因すると推定された。