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STRUCTURAL DIFFERENCES IN THE NUCLEIC ACIDS OF SOME TOBACCO MOSAIC VIRUS STRAINS*

II. DI- AND TRI-NUCLEOTIDES IN RIBONUCLEASE DIGESTS

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SUMMARY

The nucleic acids isolated from three strains of tobacco mosaic virus were digested with pancreatic ribonuclease at 23° for twelve hours at pH 7.6. The di-nucleotides, adenylyl-cytidylic acid, adenylyl-uridylic acid and guanylyl-uridylic acid and a tri-nucleotide, adenylyl-guanylyl-cytidylic acid were isolated from such digests and estimated. There was a significant difference in the amounts of these occurring in each of the digests. From the results it was concluded that the way the individual nucleotides are arranged in each of the nucleic acids of TMV, HR and M is different.

INTRODUCTION

The protein and nucleic acid components of tobacco mosaic virus (TMV) strains were analyzed with a view to establishing a chemical basis for the distinctive biological

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differences exhibited by different strains. These investigations demonstrated that the base composition of their intact nucleic acids[1,2] and that of the pancreatic ribonuclease resistant residues[3] prepared from them were essentially the same in all strains examined, while the protein composition varied from strain to strain with the exception of three pairs of strains[4].

If the biological differences exhibited by different strains are mainly due to their nucleic acid component[5,6], nucleic acids having similar base composition can still differ from each other in the way the individual nucleotides are arranged in their molecules. An attempt to probe into the arrangement of nucleotides in the nucleic acids of TMV strains by degrading them with pancreatic ribonuclease which has been shown to be a highly specific phosphodiesterase hydrolyzing only secondary phosphate esters of pyrimidine ribonucleoside 3'-phosphates[7-9], yielded interesting and promising data[10]. The pancreatic ribonuclease digests of 4 strain nucleic acids (TMV, HR, M and YA) when examined for monopyrimidine nucleotides revealed significantly greater amounts of cytidylic and uridylic acids in the digest of M strain nucleic acid than in the digests of TMV, HR and YA. This is a clear indication of a larger proportion of pyrimidine polynucleotide segments in M strain nucleic acid than in the nucleic acids of TMV, HR and YA. It was thought a further examination of these digests for di- and tri-nucleotides might give more information concerning the differences in the distribution of the nucleotides in the nucleic acids of different strains.

The present communication deals with the isolation and estimation of di-nucleotides and some tri-nucleotides in the pancreatic ribonuclease digests of TMV, HR and M strain nucleic acids. The differences in the amounts of di- and tri-nucleotides found in these digests, further demonstrate the differences in the arrangement of nucleotides in the strain nucleic acids.

MATERIALS AND METHODS

Nucleic acids were isolated from the purified preparations of each virus employing two different procedures: (1) heat denaturation procedure[11,12] and (2) detergent procedure[13,14]. Nucleic acids prepared using these two procedures will be referred to hereafter as HD and SDS preparations respectively.

Pancreatic ribonuclease used in these studies was a crystalline preparation obtained from the Worthington Biochemical Corporation, Freehold, New Jersey.

Tobacco leaf ribonuclease was prepared using the procedure of FRISCH-NIGGEMEYER AND REDDI[15].

Snake venom diesterase was prepared according to the modified acetone fractionation procedure of KOERNER AND SINSHEIMER[16], with some minor changes. 120 mg *Crotalus adamanteus* venom, obtained from Ross Allen's Reptile Institute, Silver Springs, Florida, were dissolved in 12 ml distilled water, held at room temperature for 1 h and centrifuged at 5000 rev./min at 4° for 15 min. The supernatant was adjusted to pH 4.0 with 0.2 *M* acetic acid. To 10 ml of this at 0°, 7.3 ml acetone at —20° were added. The mixture after swirling was held at 0° for 30 min and centrifuged at 5000 rev./min for 15 min at 4°. To the supernatant was added 1.4 ml acetone and held at 2° for 12 h. It was then centrifuged at 5000 rev./min for 15 min at 4°. To the supernatant was added 1.2 ml acetone, centrifuged after standing for 30 min at 0°. The final precipitate was taken up in 1 ml distilled water. When this

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preparation was tested for 5'-nucleotidase activity using adenosine 5'-phosphate as substrate neither adenosine nor inorganic phosphorus was detected. Thus the preparation appears to be free from 5'-nucleotidase activity.

Digestion of TMV-NA with pancreatic ribonuclease, isolation and estimation of di- and tri-nucleotides

TMV-NA was digested with pancreatic ribonuclease using the procedure previously described[10]. 3 mg of nucleic acid were dissolved in 0.3 ml of 0.02 *M* phosphate buffer at pH 7.6. To this was added 75 μg of ribonuclease dissolved in 0.3 ml of 0.02 *M* phosphate buffer at pH 7.6 and a few drops of chloroform to prevent microbial growth. The mixture was incubated at room temperature (23°) for 12 h.

0.1 ml of digest was placed on a large Whatman 3 mm filter paper (55 × 54 cm) at one corner at a distance of 10 cm from the top and side and dried in a current of air. The paper was placed in a Chromatocab and developed in a solvent system, containing isopropanol–water (70:30, v/v) with NH_3 in the vapour phase[7], for 72 hours at room temperature[17]. The developed chromatogram was dried at room temperature and Fig. 1a is a photograph taken according to the modified procedure of SMITH AND ALLEN[18]. This chromatographic procedure fractionates the digest into four distinct

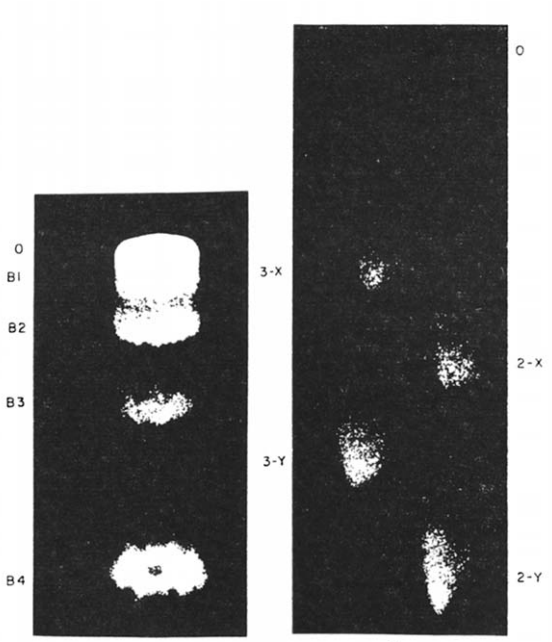

Fig. 1a. Chromatogram of 12-hour ribonuclease digest of TMV-NA at 23° and pH 7.6, developed in isopropanol-water-NH_3 solvent system for 72 hours at 23°. O, origin of the chromatogram; Band 1, a mixture of tri- to hexa-nucleotides; Band 2, a mixture of adenylyl-guanylyl-cytidylic acid and guanylyl-uridylic acid; Band 3, a mixture of adenylyl-cytidylic acid and adenylyl-uridylic acid; Band 4, a mixture of cytidylic and uridylic acids.

Fig. 1b. Electrophoresis of Bands 2 and 3 at pH 3.5 using 0.05 *M* ammonium formate buffer. 2 – X, adenylyl-guanylyl-cytidylic acid; 2 – Y, guanylyl-uridylic acid; 3 – X, adenylyl-cytidylic acid; 3 – Y, adenylyl-uridylic acid.

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bands numbered 1 to 4 in order of R_F values (Fig. 1a). The portion of the chromatogram containing bands 2 and 3 was subjected to electrophoresis at pH 3.5[7]. This procedure separated band 2 into two distinct spots, 2-x and 2-y, and band 3 into 3-x and 3-y (Fig. 1b). These were eluted by allowing distilled water to run along them. Several such eluates of each spot were combined and dried *in vacuo* at room temperature over anhydrous $CaCl_2$. The chemical nature of the substances present in each of the spots was established by using the following procedures.

2-X

(a) The dried eluate was hydrolyzed with 70–72 % $HClO_4$ and the digest after diluting with equal amount of distilled water was subjected to chromatography in isopropanol-water-HCl solvent system[19] for 18 h at room temperature. Three spots corresponding to guanine, adenine and cytosine were noticed.

(b) The dried eluate was hydrolyzed at pH 5.1 with tobacco leaf ribonuclease[20] and the hydrolysate was subjected to electrophoresis at pH 3.5[7]. Three spots corresponding to cytidylic acid, adenylic acid and guanylic acid were noticed. The molar ratio A/G/C was found to be 1.0/1.0/0.98.

All this suggests that 2-X contains a tri-nucleotide containing adenylic, guanylic and cytidylic acids. Since the tri-nucleotide was isolated from pancreatic ribonuclease digests it can be assumed that this is terminated by cytidine 3'-phosphate. The sequence of the other two nucleotides, adenylic acid and guanylic acid was established as follows.

(c) The dried eluate of 2-X was taken in 0.05 *M* veronal buffer containing 0.02 *M* Mg^{++} at pH 8.8 and was digested with snake venom diesterase. The hydrolysate was found to contain adenosine, guanosine 5'-phosphate and cytidine-3',5'-diphosphate. This reaction is illustrated in Fig. 2.

From these results it can be concluded that the tri-nucleotide present in 2-X is adenylyl-guanylyl-cytidylic acid (AGC).

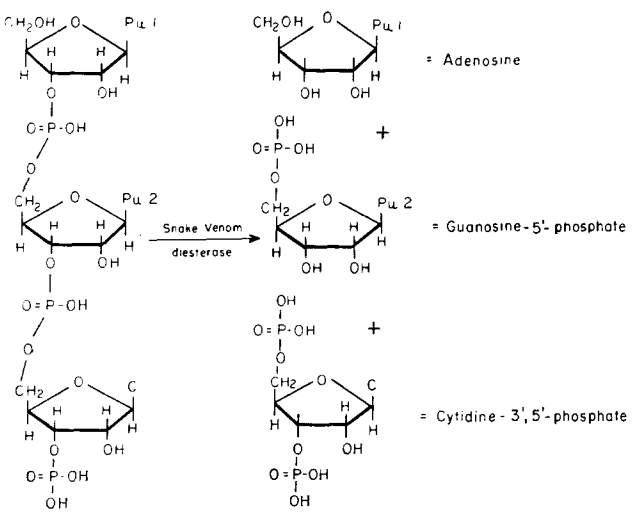

Fig. 2. Hydrolysis of tri-nucleotide present in 2 – X (Fig. 1b) with snake venom diesterase. Pu = purine (adenine or guanine); C = cytosine.

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2-Y

By using the procedures (a) and (b) evidence was obtained to show that 2-Y contained guanylic acid and uridylic acid in molar ratio of G/U = 1.0/1.01. Hence 2-Y is a guanine-uracil containing di-nucleotide. Since this was obtained from pancreatic ribonuclease digests it can be assumed that the di-nucleotide is terminated by uridine 3′-phosphate. The di-nucleotide is guanylyl-uridylic acid (GU).

TABLE I

DI-NUCLEOTIDES IN THE TWELVE HOUR PANCREATIC RIBONUCLEASE DIGESTS OF TMV STRAIN NUCLEIC ACIDS

Strain	*Type of NA preparation*	*AC*		*AU*		*GU*	
		μg/mg NA	*Average*	*μg/mg NA*	*Average*	*μg/mg NA*	*Average*
TMV	HD	37.7		44.4		48.3	
		37.7		45.6		47.8	
		41.3	39.4	45.0	44.8	47.8	47.6
	SDS	41.0		44.4		48.2	
		39.2		44.6		46.2	
HR	HD	37.0		41.4		55.6	
		35.6		40.8		55.6	
		37.0	36.5	41.4	40.8	56.7	55.1
	SDS	35.6		39.9		53.2	
		37.4		40.5		54.3	
M	HD	44.5		47.1		49.0	
		43.3		47.5		50.3	
		44.4	44.3	46.7	46.8	48.4	48.9
	SDS	44.5		45.4		49.0	
		44.8		47.4		48.0	

AC = Adenylyl-cytidylic acid; AU = Adenylyl-uridylic acid; GU = Guanylyl-uridylic acid; NA = Nucleic acid.

TABLE II

TRI-NUCLEOTIDE, ADENYLYL-GUANYLYL-CYTIDYLIC ACID IN THE TWELVE HOUR PANCREATIC RIBONUCLEASE DIGESTS OF TMV STRAIN NUCLEIC ACIDS

Strain	*Type of NA preparation*	*AGC μg/mg NA*	*Average*
TMV	HD	85.0	
		87.0	
		85.6	85.3
	SDS	84.8	
		83.9	
HR	HD	74.0	
		75.2	
		69.0	72.8
	SDS	73.6	
		72.0	
M	HD	71.8	
		74.3	
		72.9	72.6
	SDS	71.0	
		73.1	

AGC = Adenylyl-guanylyl-cytidylic acid; NA = Nucleic acid.

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3-X and 3-Y

By applying the procedures (a) and (b) it was found that 3-X and 3-Y contained di-nucleotides, adenylyl-cytidylic acid (AC) and adenylyl-uridylic acid (AU) respectively.

The amounts of these nucleotides present in the pancreatic ribonuclease digest of TMV-NA were determined spectrophotometrically and the results are given in Tables I and II.

Digestion of nucleic acids of different strains of TMV with pancreatic ribonuclease

Nucleic acids of three different strains of TMV[1,21,22] were digested with pancreatic ribonuclease according to the procedure described above. The digest was fractionated and the di- and tri-nucleotides present in the fractions were identified and estimated as described above. The results are given in Tables I and II.

RESULTS AND DISCUSSION

The hydrolysate of TMV-NA obtained by the action of pancreatic ribonuclease, when subjected to chromatography in isopropanol–water–NH_3 solvent system, resulted in the separation into four distinct bands (Fig. 1a). Band 1 contains a complex mixture of nucleotides, ranging from tri- to hexa-nucleotides (analyses of these will be reported at a later date); band 2, a mixture of a tri-nucleotide, AGC and a di-nucleotide, GU; band 3, a mixture of di-nucleotides, AC and AU; and band 4, a mixture of two monopyrimidine nucleotides, cytidylic acid and uridylic acid. In the twelve hour digest of TMV-NA only three di-nucleotides were observed and no di-nucleotide, GC could be found. The amounts of these di-nucleotides were 3.9 (AC), 4.5 (AU) and 4.8 (GU) per cent of intact nucleic acid.

These data and the specificity of pancreatic ribonuclease action permit certain conclusions to be drawn concerning the arrangement of nucleotides in the TMV-NA molecule. For di-nucleotides, AC, AU and GU to appear in the pancreatic ribonuclease digest of TMV-NA, the nucleotides, A and G must be preceded by a pyrimidine nucleotide. Thus sequences of the type PyAUR, PyACR and PyGUR (where Py is the pyrimidine nucleotide, either cytidylic acid or uridylic acid and R is any one of four nucleotides), exist in the intact TMV-NA. The sequences of the type PyGCR must not be present since the di-nucleotide, GC is not present in the complete digest of TMV-NA. Instead a sequence of the type, PyAGCR is present since a tri-nucleotide AGC is present in the ribonuclease digest. Thus guanylic acid preceded by a pyrimidine nucleotide and followed by cytidylic acid appears not to exist in TMV-NA.

In the digests of nucleic acids of HR and M, three di-nucleotides, AC, AU and GU were found. There was no di-nucleotide, GC in either of these digests. One tri-nucleotide, AGC was also isolated from these digests. However, the amounts of di-nucleotides and also tri-nucleotide occurring in the digests of nucleic acids obtained from these strains differed significantly (Tables I and II). The amounts of AC present in the digests of nucleic acids of TMV and HR differed significantly from the amount present in the digest of M strain nucleic acid. The amounts of GU and AU present in the digests of TMV and M strain nucleic acids were the same, and differed significantly from the amounts present in the digest of HR nucleic acid. The same amounts of AGC were found in the digests of HR and M strain nucleic acids while the amount present in

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TMV nucleic acid was significantly different. Since the base composition of these three nucleic acids is the same, these differences in the amounts of di- and tri-nucleotides present in the pancreatic ribonuclease digests can be attributed only to differences in the way the nucleotides are arranged in each of the nucleic acids of TMV, HR and M. The di- and tri-nucleotides appear in the pancreatic ribonuclease digest of a nucleic acid only if sequences of the type, PyPuPyR and PyPuPuPyR are present in it. The amounts of these present in the digest depend upon the number of such groups present in the intact nucleic acid. Thus the above results further demonstrate the differences in the sequential arrangement of nucleotides in the nucleic acid molecules of TMV, HR and M. These results and those reported earlier[10] clearly provide a chemical basis for the biological differences exhibited by these strains. Studies of this nature using enzymes of different specificity may reveal more differences in the sequential arrangement of the nucleotides in the strain nucleic acids. Such studies are in progress in this laboratory.

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