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CHARACTERIZATION OF RIBONUCLEIC ACIDS FROM HAPLOID AND DIPLOID STRAINS OF *PROTOMYCES INUNDATUS*

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Abstract

Total, ribosomal and soluble ribonucleic acids have been prepared from *Protomyces inundatus* by phenol method. Ridosomal ribonucleic acid contains two components, a large component with sedimentation coefficient of 25-24S and a small component with sedimentation coefficient of 18-16S. Soluble ribonucleic acid sedimentation coefficient is 4S. The nucleotide compositions of total, ribosomal and soluble ribonucleic acids have been determined. Total and ribosomal ribonucleic acids have 50% GC contents and soluble ribonucleic acids has 54% GC contents. The structure of ribonucleic acids from haploid and diploid strains is very similar.

Introduction

The fungus *Protomyces inundatus* Dangeard is a facultative parasite on *Apium nodiflorum* and causes typical infectious galls on the leaves of host plant. The life history of *P. inundatus* has been reinvestigated by Valadon, Manners & Myers (1962). This fungus grows readily in liquid culture, budding in a yeast-like manner. It is shown that the fungus exhibits simple bipolar heterothallism. Endospores of two opposite mating types are produced in the chlamydospores. The fusion of endospores of opposite mating type gives rise to diploid endospores which infect the host plant.

The behaviour of nucleic acids in non-infectious endospores of opposite mating type and infectious diploid endospores, cultured on semi-synthetic medium, is reported (Valadon, Myers & Manners, 1962). It is established that diploid endospore cultures, in spite of having double the amount of deoxyribonucleic acid (DNA) content per cell as compared to haploid mating type endospore cultures, possess the same amount of ribonucleic acid (RNA) contents per cell as haploid mating endospore cultures; in other words, the diploid endospore cell contains one half RNA per unit DNA as compared to haploid mating endospore cell. These authors suggested that DNA alone is contributed from one of the conjugants to zygote at the fusion and thus RNA contents remain at the level of the haploid cell or that both DNA and RNA are contributed from each of the conjugants and DNA undergoes an extra replication without the concomitant increase in RNA before it settles to the normal division cycle.

Venitt, Myers & Manners (1968), have studied the redeployment of RNA in zygote formed on fusion of opposite mating type endospores by labelling RNA with ^{3}H-uridine. They concluded that RNA is contributed by both the parents to the zygote and there was no reason to equate either of the mating type endospore with male or female gamete. Moreover it was documented that the RNA is distributed to the buds cutting off zygote.

It has been shown that the difference in haploid and diploid strains of *P. inundatus*, for RNA to DNA ratio, can not be ascribed to difference in nutritional physiology; because experiments indicate that the haploid and diploid strains have similar glucose yield constant, glucose saturation constant and growth rate, and can utilize various nitrogen and carbon sources for growth with equal efficiency (Ahmad, 1967).

Some physical and chemical characteristics of RNA, isolated from non-infectious mating type haploid and infectious diploid endospore cultures, are presented. Sedimentation runs demonstrate no differences in rRNA or sRNA from haploid or diploid cultures. Nucleotide composition of total RNA, rRNA or sRNA is similar for the haploid or diploid cultures.

Materials and Methods

a. Strains and culture growth

A diploid strain 2nB was derived from a fused endospore isolated from a germinating chlamydospore collected from an infected *A. nodiflorum* plant while growing wild. Two haploid strains 18+ and 2— were established from chiamydospores obtained from *A. nodiflorum* plant infected by strain 2nB under green house conditions. Strain F_2 was a diploid culture derived from a cross between 18+ and 2—, and it was incompatible with both the parents.

Cells were grown in basal medium, glucose, 10g; l-asparagine, 2g; KH_2PO_4, 1g; $MgSO_4.7H_2O$, 0.5g; Fe^{++}, 0.2 mg; Zn^{++}, 0.2 mg; Mn^{++}, 0.1 mg; biotin, 5μ g; thiamine, 100 μg; and glass distilled water to 1000 ml, (Valadon, Manners & Myers, 1962), under forced aeration, and harvested by centrifugation at a time when 5-6 g packed wet weight per litre of culture expected. The cells were washed twice with ice cold water and once with MT buffer (0.01 M $MgCl_2$, 0.05 M tris HCl, pH 7.5), and stored at —20°C until used for ribonucleic acid extraction.

b. Total ribonucleic acid extraction

Total ribonucleic acid was extracted by the method of Marcot—Queiroz & Monier (1965), with the exception that the phenol treatment was carried out at room temperature and the rest of the procedure was performed at 4°C or over ice. The cells were suspended as a thick paste in MT buffer and broken by passage through an X-press, bought from AB BIOX NACKA, SWEDEN, at—25°C under 14,000 p.s.i. About 2-3 g packed wet weight broken cells were suspended in 20 ml of MT buffer, centrifuged at 15,000 xg for 10 min and the supernantant recovered. Bentonite was added at 3.5 mg. per ml. of cell extract supernatant and then an equal volume of freshly prepared 90% phenol added and the mixture stirred at room temperature. After 15 minutes stirring, the aqueous layer separated by centrifugation. The nucleic acids were precipitated from the aqueous layer by addition of 0.1 vol of 20% sodium acetate, pH 5.0, and 2 vol of cold ethanol keeping at—20°C overnight. The nucleic acids precipitate was recovered by centrifugation and dissolved in small volume of MT buffer and reprecipitated two times; the final precipitate dissolved in 0.5 M sodium citrate, pH 5.0. Bentonite was added to the milky solution to a concentration of 3.5 mg. per ml. and centrifuged at 100,000 xg. for 45 minutes to remove polysaccharides. The clear supernatant was removed and the nucleic acids precipitated with ethanol, this precipitate was used to determine the nucleotide composition for total ribonucleic acid.

c. Ribosomal Ribonucleic acid extraction

The ethanol precipitate obtained from 100,000 xg. supernantant was dissolved in a minimum volume of 0.5 M sodium citrate, pH 5.0., and the ribosomal ribonucleic acid (rRNA) precipitated by adding 0.25 vol. of 5M NaCl and collected after 24 hr. standing at 4°C. The rRNA was washed with 2M NaCl and dissolved in 0.05 M NaCl to a concentration 5-6 mg. per ml. and stored at —20°C until required.

d. Soluble ribonucleic acid extraction

Soluble ribonucleic acid (sRNA) was extracted from intact cells by phenol treatment, Burkard *et. al.*, (1965). The packed cells were suspended in an equal volume of MT buffer and the heavy suspension mixed with an equal volume of 90% phenol, freshly prepared. The mixture was shaken vigorously for 1 hr. at room temperature and the aqueous layer removed by centrifugation at 10,000 xg. The aqueous layer removed and treated with 0.1 vol. of 20% sodium acetate, pH 5.0, and sRNA precipitate recovered and dissolved in small volume of 1 M NaCl and reprecipitated. The reprecipitation step was repeated two times and the final precipitate dissolved in 1 M NaCl and any amount of remaining insoluble material was centrifuged off at 20,000 xg. for 30 min. The sRNA solution at 3-4 mg. per ml. concentration was stored at —20°C.

e. Purity of ribonucleic acid preparations

Purity of rRNA or sRNA preparations was checked by ultraviolet spectra, which were recorded on Beckman DB recording spectrophotometer. Protein contents in the preparations were determined by the method of Lowry *et. al.*, (1951). Also sedimentation runs in analytical ultracentrifuge could tell RNA degradation in the preparations.

f. Sedimentation coefficient determination

The sedimentation coefficients of rRNA and sRNA preparations were determined using analytical ultracentrifuge Spinco Model E(Schachman, 1957; Tissieres, 1959).

g. Nucleotide composition determination

Nucleotide composition of total RNA, rRNA or sRNA was determined by alkaline hydrolysis and subsequent ion-exchange chromatography on Dowex-1-formate x8 (200-400 mesh). The RNA was dried and digested with 0.3 N KOH at 37°C for 18 hr. Unhydrolysed DNA and $K+$ were removed by precipitating with cold perchloric acid (PCA) at 4°C and the supernantant containing ribonucleotides was chromatographed on Dowex-1-formate by the procedure of Cohn (1960).

Dowex-1-chloride x8 (200-400 mesh) was purchased from Sigma Chemical Company and converted into formate form by treating the resin with 3M sodium formate containing 4% formic acid on a sintered glass funnel until no chloride was detected in the washings. After freeing the resin from chloride it was washed with water and then treated with 3 volumes of 80% formic acid to remove the remaining impurities. The resin was then washed with water until the washings were neutral.

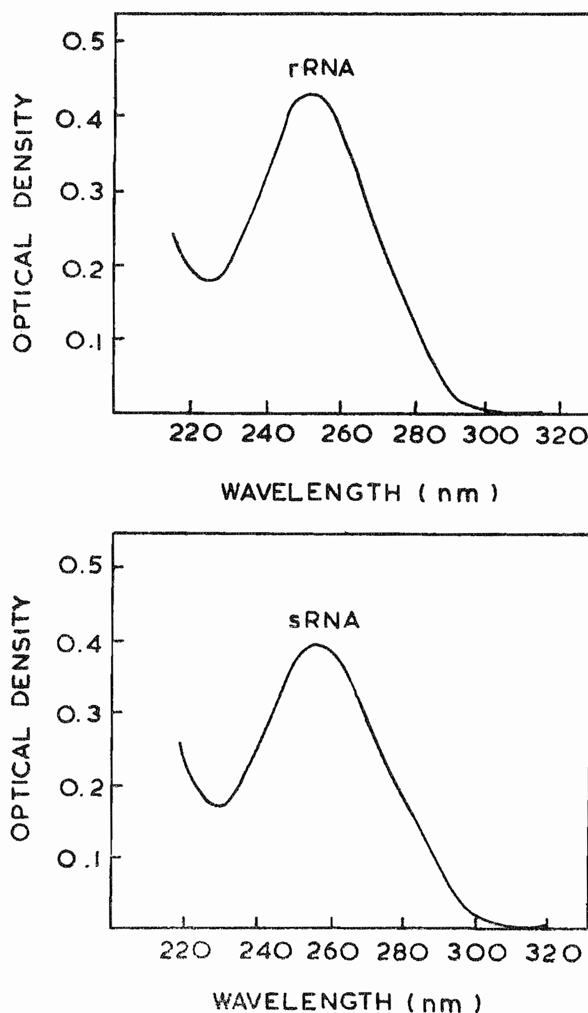

Fig. 1. Ultraviolet absorption spectra of *Protomyces inundatus* rRNA and sRNA preparations in 0.05 M. NaCl.

The KOH hydrolysate of RNA was neutralised with PCA, precipitated $KClO_4$ removed by centrifugation, and then the supernatant having ribonucleotides was diluted with water and brought to pH 9.5 with ammonium hydroxide and applied to a column of Dowex-1-formate x8 (200-400 mesh), 1 cm. in diameter and 2.1 cm. high. The ultra-violet absorbing ribonucleotides were retained as a clear zone at the top. Impurities were removed by washing the charged column with water and then with 0.005 M formic acid. Cytidylic acid, adenylic acid, uridylic acid and guanylic acid were eluted stepwise with 0.025 M formic acid, 0.15 M formic acid; 0.01 M formic acid+0.0 M ammonium formate, 0.01 M formic acid+ 0.2 M ammonium formate. Nucleotides were recognized by their characteristic absorption spectra.

Quantities of nucleotides were calculated by their molar extinction coefficients at 260 nm, either by integrating optical desnity values or from optical density values of pooled fractions. Molar extinction coefficients used were:— adenosine, $E_{260} = 1.4 \times 10^3$; cytidylic acid, $E_{260} = 6.8 \times 10^3$, adenylic acid, $E_{260} = 14.2 \times 10^3$; uridylic acid, E260 = 9.8×10^3, guanylic acid, $E_{260} = 11.8 \times 10^3$; these being the values appropriate for pH's used, (Zubay, 1962).

Results

The absorption spectra for rRNA and sRNA were recorded (Fig. 1). It was not possible to distinguish among the ultraviolet spectra of rRNA or sRNA preparations made from different strains of *P. inundatus*, however, the analysis of utlraviolet absorption spectra could differentiate rRNA from sRNA (Table 1). For rRNA the ratio OD_{258}nm to OD_{280}nm ranged between 2.27-2.30, while this ratio for sRNA was 2.00-2.04. Protein contents were less than one percent in all the RNA prepartions.

Sedimentation runs for rRNA preparations characterized two components. The schlieren diagram showed a fast moving component of 25S and a second component 18S (Fig. 2.A.) Sedimentation with ultraviolet optics also showed two components for rRNA, however, the large component has sedimentation coefficient of 24S and the small component has sedimentation coefficient of 16S (Fig. 2.B). The sedimentation coefficient for sRNA was 4S (Fig. 2.C). The RNA preparations from haploid and diploid strains have similar sedimentation properties.

TABLE 1. Absorption spectrum analyses and protein contents of purified ribonucleic acids.

Preparations	Absorption spectrum analyses	Protein contents percentage
rRNA	$\frac{258\ nm}{230\ nm} = 2.37\text{-}2.43$	∠ 1
	$\frac{258\ nm}{280\ nm} = 2.27\text{-}2.30$	
sRNA	$\frac{258\ nm}{230\ nm} = 2.33\text{-}2.34$	∠ 1
	$\frac{258\ nm}{280\ nm} = 2.00\text{-}2.04$	

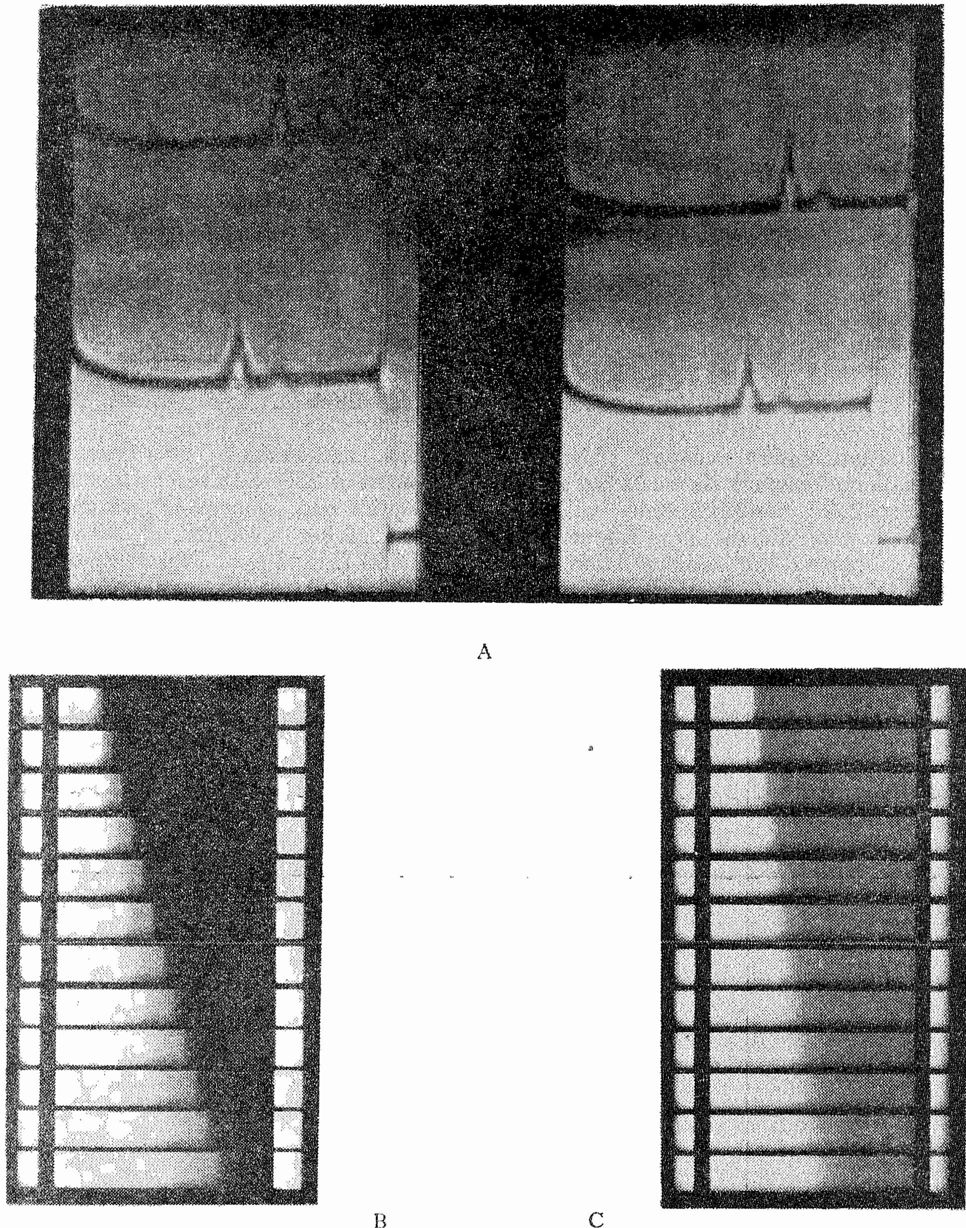

Fig. 2. Schlieren diagram of rRNA from *Protomyces inundatus* strain 2-(upper pattern) and strain 2nB (lower pattern), at 1 mg. per ml. concentration in 0.05 M. NaCl. The centrifugation was at 59,780 rpm at 20^{0}C. Sedimentation proceeds from right to left and the pictures were taken at 2 minutes interval, bar angle 50^{0} and 45^{0} respectively and a D (2f E double sector) cell was used. B. Sedimentation diagram of rRNA, from *P. inundatus* strain 18+ with ultraviolet optics. The rRNA concentration was 40ug per ml in 0.05m NaCl. The centrifugation was at $59{,}780^{0}$ pm at 20^{0}c, and sedimentation proceeds from left to right. The pictures were taken at 8 minutes intervals and a (Kel F single sector) cell was used. C. Sedimentation diagram of sRNA from *P. inundatus* at 40 ug per ml concentration in 0.05 M Na Cl, at 59,780 rpm at 20^{0} C, and sedimentation proceeds from left to right. The pictures were taken at 8 minutes intervals and a (Kel F single sector) cell was used.

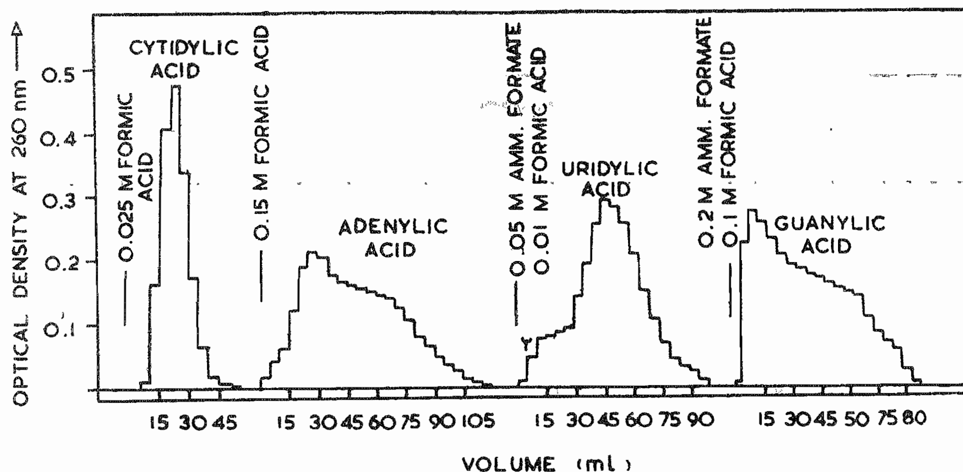

Fig. 3. Ion exchange separation of nucleotides of alkaline hydrolysate of about 3mg rRNA of *P. inudatus*, on Dowex-1, x8 (formate form); column size was 1 cm in diameter and 2.1 cm high. Flow rate was about 1.5 ml per minute.

The nucleotide compositions of total RNA, rRNA, and sRNA are presented in Table 2; and the error is expressed as the average deviation from the mean of four strains. Pseudouridylic acid (Y) eluted just before uridylic acid (Fig. 3), and it was pooled with uridylic acid. The nucleotide composition of total RNA evidently reflects the composition of rRNA in 50% GC contents and in approximate equality of purines and pyrimidines—G+A = C+U. Ribosomal RNA composition observed approximate equality of 6-keto groups with the number of 6-amino groups—G+U = A+C. In sRNA, the 54% GC contents were different from that of rRNA, mainly because cytidylic acid contents were higher whereas the adenylic acid and uridylic acid were lower. Haploid and diploid strains contained the same type of total, ribosomal or soluble RNA.

Discussion

The values of sedimentation coefficients of ribosmal RNA of *P. inundatus* are 25S and 18S for the large and small components respectively, in 0.05 M NaCl, with schlieren optics, however, ultraviolet optics gave lower values of 24S and 16S. These values are in agreement with the values obtained for different species of fungi (Taylor *et. al.*, 1967). It may be noted that the values of the sedimentation coefficients of RNA depend substantially upon the ionic conditions in solutions, impurities of bound ions of divalent and polyvalent metals in the preparations and measurements made with either schlieren optics or ultraviolet optics. The exact values of sedimentation coefficient of ribosomal RNA's of different species of fungi are about 23.4±0.5 and 16.4±0.2, in 0.1 M NaCl and 0.01 M EDTA in presence of 25 ug/ml polyvinyl sulphate pH 4.5., and these values increase to 30S and 20S in 0.01 M $MgCl_2$ (Taylor *et. al.*, 1967). In bacteria, the values of the sedimentation coefficients of rRNA are about 20S and 15S,

TABLE 2. **Base composition of total RNA, ribosomal RNA, and soluble RNA from haploid and diploid strains of** *Protomyces inundatus*.

Strains	Nucleotide contents, mole%				%GC	(G+A)/(C+U)	(G+U)/(A+C)
	A	C	G	U			
				Total RNA			
18+	22.34	20.83	28.97	27.86	49.80	1.05	1.32
2—	22.56	20.78	29.69	27.27	50.47	1.09	1.31
2nB	21.68	20.67	29.89	28.17	50.56	1.06	1.37
F2	22.71	22.19	27.61	27.49	49.80	1.01	1.23
Mean	22.32	21.12	29.04	27.70	50.16	1.05	1.31
S.E.	±0.23	±0.36	±0.52	±0.20			
				Ribosomal RNA			
18+	25.34	22.41	26.27	25.98	48.68	1.13	1.09
2—	25.63	22.46	25.04	26.84	47.50	1.03	1.08
2nB	23.66	22.21	27.75	26.36	49.96	1.06	1.18
F2	25.15	23.07	27.12	25.64	50.19	1.07	1.09
Mean	24.95	22.54	26.54	26.21	49.08	1.06	1.11
S.E.	±0.44	±0.19	±0.59	±0.26			
				Soluble RNA			
18+	20.38	26.15	27.60	25.87	52.02	0.92	1.15
2—	20.35	27.21	29.67	22.75	56.88	1.00	1.10
2nB	20.27	25.54	28.84	25.34	54.38	0.97	1.18
F2	20.80	25.34	28.45	25.29	53.88	0.97	1.15
Mean	20.45	26.08	28.64	24.81	54.72	0.96	1.15
S.E.	±0.12	±0.41	±0.43	±0.70			

A—adenylic acid, C—cytidylic acid, G—guanylic acid,
U—uridylic acid and also includes pseudouridylic acid.
S.E.—Standard error.

in 0.1M NaCl with 0.01 M EDTA in presence of 20 ug/ml polyvinyl sulphate pH 4.5, at low RNA concentration with ultraviolet optics, however, it is shown that the values of sedimentation coefficients are 23S and 17S, at high RNA concentration and extrapolation to zero concentration, in an analogous solvent of phosphate buffer with ionic strength 0.1M, 0.01 M EDTA, with schlieren optics (Spirin & Gavrilova, 1969).

The nucleotide composition of total RNA and ribosomal RNA is nearly the same, this indicates that, as in *Neurospora crassa* and *Aspergillus niger* (Henney & Storck, 1963; Moyer & Storck, 1964); the ribosomal RNA accounts for the bulk of the cell RNA, and like in most fungi G+C = A+U (Uryson & Belozersky, 1960; Vanyushin, Belozersky & Bogdanova, 1960).

In *P. inundatus*, the soluble RNA has 54% GC contents, higher than the total RNA or rRNA GC contents, and this increase is mainly due to high proportion of cytidylic acid. This result is in agreement with those reported in literature for yeast, bacteria and animal tissue (Osawa, 1960) and in *N. crassa* and *A. niger* (Henney & Storck, 1963; Moyer & Storck, 1964).

No differeneces in the RNA from haploid and diploid strains are found; the molecular size of rRNA components is identical and also the nucleotide compositions of total RNA, rRNA and sRNA are almost the same. The difference in the RNA contents per unit DNA in haploid and diploid strains might be due to regulation of rate of RNA synthesis at the genetic level. It is shown that in *Drosophila melanogaster* (Ritossa & Spiegelman, 1965), one to four loadings of ribosomal DNA; and in *Xenopus laevis* (Wallace & Birnstiel, 1966), one to two loadings of ribosomal DNA control the levels of ribosomal RNA contents in the cell. The presence of any special RNA in substantial quantity in diploid cells in the light of this investigation, can be safely ruled out.

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